DETERMINATION OF FACTORS LIMITING ENZYMATIC HYDROLYSIS OF THE *CHLORELLA SOROKINIANA* CELL WALL

by

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

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So much of what I have learned has not been from books or in the classroom, but through special people who were willing to share their experience.

To my teachers

ACKNOWLEDGMENTS

I am grateful to Dr. Robert R. Schmidt for providing the opportunity to conduct this research project in his laboratory, and for having patience and commitment to guide the project through to its completion. I thank my supervisory committee members, Dr. Henry C. Aldrich, Dr. Robert J. Ferl, Dr. John E. Gander, and Dr. L. Curtis Hannah, for their important contributions towards the development and execution of this research project.

Dr. Henry C. Aldrich, Donna Williams and Lorraine McDowell provided expertise, supplies, and undying patience contributing towards "just one more" electron microscopy experiment. Dr. Dave Powell provided generous help with the elemental analysis, infrared spectroscopy, and interpretation of data. Dr. Marian Busko provided assistance with the NMR experiments, and Dr. Chris West was helpful with the carbohydrate analysis data.

This research was enhanced by the generosity of Dr. Ryzard Brzezinski of the Universite de Sherbrooke for supplying the *Streptomyces lividans* culture, Dr. Robert Lamoreaux of Sandoz, Agro, Inc., for providing Norflurazon, and Dr. Dennis Dunphy of HybriTech Seed International, Inc., for providing MON-20763.

Assistance in the preparation of this manuscript was kindly supplied by Marc Adkins, Jill Cocanougher, and Dr. John van Hooke.

I thank my colleagues, Waltraud I. Dunn, Dr. Philip Miller, and Richard Hutson, for their assistance and friendship. I wish to thank Dr. James E. Maruniak for always being there with helpful suggestions and support. I thank my friends Peter Gomez, Lorraine Yomano, Louise Monroe, and Joy Doran for valuable encouragement.

Completion of this degree would not have been possible without support and love provided by my family. I wish to thank my mother Barbara Phipps for support and understanding and my father Gerry Russell for "fatherly" advice. I thank my sisters, Barbara Campbell, Donna Cooper, Gwen Trufant, and Mary Dougherty-Hunt, (who may as well be my sister) for always being there for me.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS x

ABSTRACT xi

INTRODUCTION 1

LITERATURE REVIEW 4

MATERIALS AND METHODS..................................27
- Culture Conditions 27
- Source of *Chlorella* Cultures 28
- Preparation of Samples for Transmission Electron Microscopy 28
- Preparation of Samples for Scanning Electron Microscopy 29
- Determination of Cell Wall Thickness 30
- Purification of *Chlorella* Cell Walls 30
- Dry Weight Determinations 31
- Instruments Used for Spectroscopic Analyses 31
- Extraction of Hydrolyzable Carbohydrates from *Chlorella* Walls 32
  - Acid Hydrolysis of Cell Walls 32
  - Alkaline Hydrolysis of Cell Walls 34
- Protein and Amino Acid Analysis of Purified Cell Walls 35
- Acetolysis 35
- Assessment of Cell Wall Autofluorescence 36
- Determination of Cell Wall Solubility in Phosphoric Acid 37
- Phloroglucinol Assay for Lignin 37
- Analysis of Alkaline Extracts for the Presence of Phenolic Compounds 37
- Determination of Cell Wall Solubility in Chromium Trioxide 38
- Preparation of Cell Wall Degrading Enzymes 38
  - Enzymes Tested 38
  - Growth of *S. lividans* pRL207 for Chitosanase Production..................................39
  - Purification of Chitosanase 40

    - Chitosanase Assay 41
    - Production of *C. sorokiniana* Cell Homogenate for Cell Wall Degradation 41
  - Treatment of Whole Cells with Cell Wall Degrading Enzymes 42
  - Treatment of Purified Substrates with Cell Wall Degrading Enzymes.................................44
  - Treatment of Thin Sections with Polysaccharide Degrading Enzymes 45
  - Attempts to Induce the Accumulation of Ketocarotenoids in *C. sorokiniana* and *C. fusca* Cells................45
  - Determination of the Effects of Norflurazon on Growth Rate, Carotenoid Content, and Percentage of Acetolysis Resistant Cell Wall Fraction of *C. sorokiniana* 46
  - Determination of the Effect of Growth in MON-20763 on *Chlorella* Cells 47
  - Production and Screening of Cell Wall Defective Mutants 47

- RESULTS 51

  - *C. sorokiniana* Cell Wall Characterization 51
    - Polysaccharide Analysis 51
    - Protein and Amino Acid Analyses 65
    - Analyses for Other Cell Wall Compounds 67
      - Sporopollenin 67
      - Phenolic compounds 76
      - Acetolysis-resistant portion of the *C. sorokiniana cell* wall 79
  - Effect of Hydrolytic Enzymes on *C. sorokiniana* Cell Wall 80
    - Effect of Enzymes on Whole Cells 80
    - Effect of Enzymes on Purified Walls 86
    - Effect of Enzymes on Thin Sections of Cells 92
  - Attempts to Disrupt *C. sorokiniana* Cell Wall Biosynthesis 92
    - Attempts to Induce Ketocarotenoid Synthesis in *C. sorokiniana* and *C. fusca* Cells....................92
    - Effect of Norflurazon on *C. sorokiniana* Cell Walls ..97
    - Effect of MON-20763 on Cell Wall Synthesis.........101
  - Creation, Selection, and Characterization of Cell Wall Defective Mutants 109

- DISCUSSION 129

- LIST OF REFERENCES 144

- BIOGRAPHICAL SKETCH 153

LIST OF TABLES

Table page

1. GC-MS determination of constituent monosaccharide composition of the *C. sorokiniana* cell wall 55

2. Colorimetric analyses of acid hydrolysates of *Chlorella* cell walls 57

3. Elemental analysis of chitosan and *C. sorokiniana* cell wall residues 64

4. Amino acid composition of *C. sorokiniana* cell wall proteins 66

5. Acetolysis and phosphorolysis of purified *Chlorella* cell walls 68

6. Percent degradation of purified cell walls subjected to enzymatic digestion 88

7. Effect of norflurazon on growth rate, total carotenoid content, and percentage of acetolysis-resistant wall fraction of *C. sorokiniana* cells......100

8. Identification of carotenoids from *C. sorokiniana* using thin layer chromatography and absorption spectrum analysis 102

9. Comparison of *C. sorokiniana* wild type and mutant TFA-extractable cell wall monosaccharides 116

10. Comparison of *C. sorokiniana* wild type and mutant HCl-extractable cell wall monosaccharides 121

11. Comparison of *C. sorokiniana* wild type and mutant total extractable cell wall monosaccharides ..125

12. Comparison of *C. sorokiniana* wild type and mutant cell wall protein concentration 127

13. Comparison of *C. sorokiniana* wild type and mutant cell wall protein amino acid compostion 128

LIST OF FIGURES

Figure page

1. Identification of the monosaccharides released through acid hydrolysis of the *C. sorokiniana* cell wall 54

2. Proton-decoupled [^{13}C] NMR profile of the ethanol precipitate of an alkaline-soluble fraction of the *C. sorokiniana* cell wall.......................59

3. IR spectra of chitosan and residue remaining after alkaline extraction of the *C. sorokiniana* cell wall 63

4. Acetolysis resistant residues of *C. sorokiniana* and *C. fusca* cell walls...................70

5. Transmission electron micrographs of *C. sorokiniana* and *C. fusca* cell walls................73

6. Autofluorescence of purified *Chlorella* cell walls 75

7. IR spectra of purified *C. sorokiniana* cell walls 78

8. IR spectra of *C. sorokiniana* alkaline extracted cell wall residue and alkaline extracted cell wall residue after subsequent acetolysis treatment 82

9. TEM and SEM of *C. sorokiniana* cells untreated and treated for 16 h with CHP 85

10. Kinetics of degradation of substrates by chitosanase from *S. lividans* pRL207 90

11. Measurement of the enzymatic release of compounds containing primary amines from control substrates and the *C. sorokiniana* cell wall 94

12. TEM of thin sections of *Chlorella* cells treated with CHP after fixation and embedding of cells in Spurr's plastic 96

13. Growth of *C. sorokiniana* cells in cultures containing norflurazon 99

14. Growth of *C. sorokiniana* and *C. fusca* cells in cultures containing MON-20763 104

15. Morphology of *C. sorokiniana* and *C. fusca* cells grown in cultures containing MON-20763 106

16. Wall morphology of *C. sorokiniana* and *C. fusca* cells grown in MON-20763 with and without subsequent CHP treatment 108

17. Kill curve of *C. sorokiniana* cells exposed to UV light 111

18. Growth of wild type and mutant *C. sorokiniana* cells 114

19. Comparison of monosaccharide composition of TFA hydrolysates of wild type and mutant *C. sorokiniana* cell walls 118

20. Comparison of monosaccharide composition of HCl hydrolysates of wild type and mutant *C. sorokiniana* cell walls 120

21. Comparison of monosaccharides in total acid hydrolysates of wild type and mutant *C. sorokiniana* cells walls 124

LIST OF ABBREVIATIONS

CaMV	cauliflower mosaic virus
CHP	cellulase, hemicellulase, and pectinase
GDH	glutamate dehydrogenase
ICBR	Interdisciplinary Center for Biotechnology Research
MWCO	molecular weight cut off
ND	not determined
NOS	nopaline synthase
NP-40	Nonidet P-40
ppm	parts per million
RG-I	rhamnogalacturonan I
RG-II	rhamnogalacturonan II
SEM	scanning electron microscopy
TEM	transmission electron microscopy
TMS	tetramethylsilane
TSP	3-(trimethylsiayl)-propionate
TSB	tryptic soy broth

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

DETERMINATION OF FACTORS LIMITING ENZYMATIC HYDROLYSIS OF THE *CHLORELLA SOROKINIANA* CELL WALL

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December, 1995

Chairman: Robert R. Schmidt
Major Department: Microbiology and Cell Science

The nature of resistance of the *Chlorella sorokiniana* cell wall to enzymatic degradation preventing protoplast formation was evaluated. The cell wall of this alga was analyzed using biochemical, microscopic, and mutagenic techniques. The cell wall was shown to be composed of approximately 83% carbohydrate and 17% protein. Using GC/MS, the major monosaccharides identified in cell wall 2 N TFA and 6 N HCl hydrolysates were rhamnose, glucuronic acid, galactose, xylitol, and mannose. Using NMR spectroscopy of 2 N NaOH cell wall hydrolysates, the presence of polysaccharides containing xylitol and at least three other monosaccharides was confirmed. NMR spectroscopy also identified polysaccharides containing β-1,6-glycosidic linkages and hydroxymethyl groups. Cell wall proteins contained only 0.02 mol% of hydroxyproline. Glycine and alanine were at slightly higher concentrations than other amino acids. Glucosamine was present in the 6 N HCl hydrolysate of the wall indicating the presence of chitosan. Compounds such as lignin, other phenolic compounds, and sporopollenin, that give some cell walls resistance to enzymatic degradation, were not found in the *C. sorokiniana* wall. Much of resistance of the cell wall to acetolysis is due to the high concentration of polysaccharides containing rhamnose. Despite severe disruption of the *C. sorokiniana* mother cell wall by growth of cells in MON-20763, a pollen biosynthesis inhibitor, the cell wall was not degraded in a solution containing cellulase, hemicellulase, and pectinase. Three cell wall defective mutants were isolated. The concentration of total cell wall protein and certain monosaccharides in cell wall acid-hydrolysates varied among mutant and wild type strains. In cell walls of each mutant, glucosamine concentration was similar to that of the wild type. Using a mixture of cellulase, hemicellulase, and pectinase, osmotically labile cells could not be prepared from any of the mutant strains.

INTRODUCTION

The eucaryotic microalga, *Chlorella*, has long been studied as a model organism for elucidation of biochemical events such as photosynthesis, transport, and nutrient metabolism. For several reasons, *Chlorella* is an ideal model organism. Large quantities of *Chlorella* cells can be cultured in days rather than weeks or months required for higher plants. Biochemical studies with *Chlorella* are not complicated by the presence of multiple cell types as in higher plants. Many species of *Chlorella* can be cultured as synchronous, biochemically homogeneous, cells.

Some of these same characteristics make *Chlorella* a useful organism for industrial production of metabolites (Millis et al., 1988; Running et al., 1994). The scope of its industrial value is limited by the difficulty of genetic manipulation of this asexual microalga. Stable transformation in *Chlorella* cells has not been achieved. Transient expression of firefly luciferase has been demonstrated in *C. ellipsoidea* protoplasts. Transformation of *C. ellipsoidea* was achieved using PEG/$CaCl_2$-mediated transfer of a plasmid containing the luciferase gene flanked by a CaMV promoter and a NOS terminator (Jarvis and Brown, 1991).

Research on *C. sorokiniana* would be enhanced by development of a transient expression assay. In this laboratory, the *C. sorokiniana* gene encoding an NADP-specific glutamate dehydrogenase has been studied extensively. The promoter of this gene may be regulated, either directly or indirectly, by concentration of ammonium (Cock et al., 1991). Plasmids could be constructed to test the effect of different promoter regions on reporter gene transcription. The level of reporter gene expression could be determined using *Chlorella* cells transformed with the plasmid constructs.

Many DNA transformation methods are designed to transfer DNA into a low percentage of a total cell population. Low transformation frequency is useful for selection of stable transformants, but not practical for assessment of transient expression. Transfer of plasmids into protoplasts using electroporation or PEG/$CaCl_2$ results in relatively high transformation frequency. In *C. sorokiniana* cells, development of a transient expression assay is limited by inability to prepare protoplasts from this alga. Production of protoplasts would also allow for more efficient isolation of its organelles for other types of experiments.

This study was designed to increase an understanding of the cause(s) of resistance of the *C. sorokiniana* wall to enzymatic digestion. This objective will be accomplished by (i) biochemical and morphological analysis of cell wall composition and structure, (ii) characterization of the effects of metabolic inhibitors on cell wall synthesis, and (iii) isolation and characterization of cell wall defective mutants.

LITERATURE REVIEW

General Cell Wall Composition

Four of the five kingdoms of life, Monera, Protista, Fungi, and Plantae, are dominated by organisms with cell walls. Only the kingdom Animalia is dominated by walless members. Since most eucaryote walls are fibrillar and polysaccharide-based and most procaryote walls are non-fibrillar and peptidoglycan-based, it is likely eucaryotic and procaryotic cell walls have evolved independently. Cell walls appeared early in evolution, most likely to enable the protoplast to maintain turgor pressure to allow for increased metabolic activity. In more recent evolutionary time, cell walls developed to provide protection to the protoplast from environmental damage and attack by pathogens. Cell walls have also enabled some single-celled organisms to occupy novel ecological niches by evolving into large multicellular forms (Ruiz-Herrera, 1992).

Most eucaryotic cell walls are composed primarily of polysaccharides, but also possess significant concentrations of protein and other compounds. Carbohydrate structure and biosynthesis are among the most complex of the biological molecules. In addition to being the primary structural components of the cell wall, some wall polysaccharides are involved in cell/cell recognition and gene regulation (McNeil et al., 1984). Protein, usually present as glycoprotein, is the second most common cell wall constituent. Some of the many functions of cell wall glycoproteins include cell structure, growth, recognition, and defense. In rare cases, protein replaces polysaccharide as the primary cell wall constituent. Despite polysaccharide and protein making up the bulk of all cell walls, additional compounds, such as lignin and other phenolic compounds, esters, lipids, silica, and pigments such as melanin and carotenoids add to variation in cell wall structure and function (Bartnicki-Garcia, 1984; Fry, 1986).

Plant Cell Wall Composition

The primary wall of a higher plant cell is about 0.1 µm thick. The fibrillar or rigid portion of a wall consists of cellulose. Cellulose, the most abundant organic compound in the world, comprises up to 30% of a primary wall. It is an unbranched polymer of β-1,4-glucose consisting of between 500 and several thousand glucose units. The glucose polymers have a parallel arrangement and are specifically hydrogen bonded to each other, forming microfibrils. Microfibrils are tightly packed in a crystalline arrangement. Cellulose microfibrils are synthesized at the plasma membrane and lie in the plane of the cell surface (McNeil et al., 1984; Read and Delmer, 1991).

Hemicellulose and pectin constitute up to 60% of most walls and compose a nonfibrillar or gelatinous matrix in which the cellulose microfibrils are embedded. Matrix polysaccharides are synthesized intracellularly and are exported to the wall in Golgi-derived vesicles. Cellulose microfibrils are subsequently deposited into the gel-like matrix (Northcote, 1991).

Depending upon composition of the polysaccharide backbone, most hemicelluloses are grouped as xylans, xyloglucans, or glucans. Xylans, polymers of β-1,4-xylose with branches of arabinose and glucuronic acid molecules, are more prevalent in grasses than they are in dicot plants. Also in grasses, ester groups of ferulic, coumaric, and hydroxybenzoic acids may be linked to xylan monosaccharides. The number of side chains varies among xylans. Xyloglucans are more prevalent in dicots than in grasses. They have a β-1,4-glucose (cellulose) backbone; most side chains consist of a single xylose residue. In addition to the xylose residue, other xyloglucan side chains have galactose, arabinose, or galactose and fucose residues. Side chains occur at different frequencies among xyloglucans. Glucans are unbranched polymers of both β-1,3- and β-1,4-glucose. They are abundant in grasses and are found infrequently, if at all, in dicots. Hemicellulose is hydrogen-bonded to cellulose microfibrils, and it likely has a structural as well as a regulatory role in the plant cell wall (McNeil et al., 1984, Fry, 1988).

Pectin is a polymer of polyuronic acid and can be present in a single cell in both smooth (unbranched) and hairy (branched) forms. Smooth pectins consist of stretches of galacturonic acid residues occasionally interrupted by rhamnose residues. Hairy pectins have backbones rich in rhamnose and galacturonic acid and side chains that vary considerably among pectins. Side chains of hairy pectins rhamnogalacturonans RG-I and RG-II can be composed of any of twelve different sugars, including rhamnose, arabinose, fucose, galactose, methylfucose, and glucuronic acid. The pectic polysaccharides vary in their extractability from walls. Hairy polysaccharides are more resistant to enzymatic degradation by pectinase than are smooth pectins. Pectins help maintain structure of the cell wall and possibly other functions within the growing plant cell wall (Chambat and Joseleau, 1980; Blaschek et al., 1981; McNeil et al., 1984; Fry, 1988).

Plant cell wall proteins constitute about 10% of the wall dry-weight and can be either tightly or loosely associated with the cell wall polysaccharides. Loosely associated cell wall proteins can be extracted with detergents, salts, or cold aqueous acids and alkalis. Tightly associated proteins become insoluble in the wall and are not easily extracted.

The most abundant and well studied cell-wall proteins fall into five classes: extensins, glycine-rich proteins, proline-rich proteins, solanaceous lectins, and arabinogalactan proteins. Of these protein classes, only glycine-rich proteins are not hydroxyproline-rich glycoproteins. The functions of many of these structurally characterized proteins are unknown. There is evidence for some functions such as maintenance of wall structure, wound healing, plant defense, pathogen immobilization, and cell-cell interactions. Finally, there are some cell wall proteins that do not fit into any of the five classes; many of these proteins are enzymes that degrade cell walls (Showalter, 1993).

Other plant cell wall components, most notably lignin and cutin, render cells with unique properties. Because of its high concentration in secondary walls, lignin is the second most abundant organic molecule on earth. Lignin, a polymer of phenylpropanoid units, is also found, to a lesser extent, in primary cell walls. It functions in both cell structure and defense against pathogen invasion. Cutin, a polyester of hydroxy fatty acids, is associated with some epidermal cells and protects the plant from water loss and other environmental stresses (Fry, 1988).

Fungal Cell Wall Composition

The fibrillar portion of the fungal wall may also consist of β-glucans (β-1,3-glucose, β-1,4-glucose, and β-1,6-glucose), α-1,3-glucans, and chitin (β-1,4-N-acetylglucosamine). Typically, the rigid portion of a fungal wall consists of either glucan or chitin. Some fungal species, particularly the Chithridiomycetes, have substantial amounts of both cellulose (β-1,4-glucose) and chitin in the wall. Chitin, which is not found in higher plant walls, composes up to 18% of some fungal walls. Chitosan, a β-1,4-glucosamine polymer with varying degrees of N-acetylation, is also associated with some fungal rigid walls. Ruiz-Herrera (1992) speculates that it is not likely that chitosan is present in these walls in the crystalline form as is chitin and glucan. The role of chitosan in rigid wall structure is unclear, although it may contribute to the resistance of the wall to degradation by pathogens. The *Saccharomyces cerevisiae* spore wall has about 10% dry weight chitosan. Chitosan is located in a layer internal to a protein layer and external to a glucan/mannan layer. This wall is highly resistant to enzymatic degradation (Briza et al., 1988). Chitosan is polycationic under physiological conditions and may associate with polyanionic glucuronic acid in the wall. Many fungal wall polysaccharides are characteristic of specific fungal groups. Polyuronides are common in fungi and consist mostly of glucuronic acid as opposed to galacturonic acid in higher plants. Different heteropolysaccharides have been described in fungi. Many polysaccharides are rich in glucose, mannose, galactose, and xylose; heteropolysaccharide composition is species-specific (Ruiz-Herrera, 1992).

As is the case in plant walls, protein concentration in fungal walls is second to that of carbohydrates. Fungal walls consist of 3%-20% protein. Hydroxyproline is not typically found in fungal walls. Protein profiles in fungi are extremely complex; attempts are being made to group fungal wall proteins into families of similar proteins.

Other components of some fungal walls include lipids, melanins (up to 21% in some spore walls), and sporopollenin (Ruiz-Herrera, 1991).

Cell Wall Composition of the Green Algae

Algal cell walls have the basic wall structure shared by plant and fungal walls; they are composed of microfibrillar polysaccharides embedded in a nonfibrillar polysaccharide matrix. Microfibrils often consist of cellulose, although, some algae have β-1,4-mannans, β-1,3-xylans, and chitosan instead of cellulose (Takeda and Hirokawa, 1984; Vian and Reis, 1991). Like fungi, algal walls have only a primary wall. Generalizations about hemicellulose structure in algae have not been made as they have in plants.

Green algae commonly possess glycoprotein-rich cell walls. Cell wall proteins of some green algae are particularly rich in the amino acids hydroxyproline, glycine, and alanine (Voigt et al., 1994). The *Chlamydomonas reinhardtii* vegetative cell wall is unique in that it lacks structural polysaccharides and consists solely of hydroxyproline-rich glycoproteins (Roberts, 1974). Hydroxyproline concentration was determined in the alkali-insoluble portion of some *Chlorella* cell walls; *C.*

ellipsoidea C-87 has 9.3 mol% hydroxyproline and three other strains of *C. ellipsoidea* (one later reclassified as *C. vulgaris*) have no hydroxyproline (Takeda and Hirokawa, 1984).

Some algae possess additional cell wall compounds such as ketocarotenoids, sporopollenin, hydrocarbons, and lignin-like compounds (Atkinson et al., 1972; Gunnison and Alexander, 1975; Berkaloff et al., 1983). Lignin is not typically associated with algae, although lignin-like compounds were identified in *Staurastrum* and *Coleochaete* (Chlorophyta, class Charophyceae), where they are likely to function as an antimicrobial agents (Alexander and Gunnison, 1975; Delwiche et al., 1989).

Taxonomy of *Chlorella*

Chlorella, a eucaryotic microalga, is classified with the Protista and, since it has chlorophyll a and b and stores carbon in the form of starch, is a member of the Chlorophyta. It divides with the aid of a phycoplast and thus is further classified in the class Chlorophyceae. *Chlorella* is unicellular and nonmotile, and possesses a single cup-shaped chloroplast. Diameter of an average *Chlorella* cell is about 3-5 µM. *Chlorella* cells divide asexually by enlargement followed by chloroplastic and nuclear divisions. Depending upon light and nutrition, a cell can divide into two, four, eight, or sixteen progeny. Cell walls form between developing progeny which are encased within the wall of the original (or mother) cell wall. The progeny, or daughter cells, are released from the surrounding mother wall most likely by both enzymatic and mechanical means. Cell wall autolytic enzymes have been characterized from several species of *Chlorella*.

Because of the morphological simplicity of the algae, taxonomic classification of *Chlorella* species is difficult. This point is well illustrated in a study in which the biochemical characteristics were tested on 58 strains of *Chlorella* from the Culture Collection of the University of Texas at Austin (Kessler and Huss, 1992). Only 17 of the 58 strains had been properly classified. The earliest attempts to group *Chlorella* species were made using approximately 13 biochemical characteristics. Chemical characterization of traits including hydrogenase activity, synthesis of secondary carotenoids, and pH, salt, and temperature limitations on growth has been used to identify *Chlorella* species. Despite the large number of biochemical tests, some strains still can not be unambiguously classified. Usefulness of chemical characterization is limited by the large number of characteristics that need to be analyzed. *C. sorokiniana* is characterized biochemically by hydrogenase activity during anaerobiosis, nitrate reduction, lack of secondary carotenoids produced under nitrogen deficiency, and growth at 38°C (Kessler, 1978; Kessler and Huss, 1992).

Cell wall composition is also useful in determination of taxonomic relatedness of *Chlorella* species. It is critical that characteristics used for taxonomic purposes be constant throughout the cell cycle of the organism. *C. ellipsoidea* C-27 maintains a constant qualitative cell wall composition throughout its cell cycle. Rigid-wall carbohydrate concentration also remains constant throughout the cell cycle; matrix carbohydrate concentration increases in proportion to the growing cell surface (Takeda and Hirokawa, 1978).

Based upon cell wall morphology, *Chlorella* species have been divided into three groups. Type-1 walls have two layers, a thick inner cellulosic layer and thin outer trilaminar layer containing sporopollenin. *C. fusca* has a typical type-1 wall. Type-2 walls also have two layers, a thick cellulosic inner layer and a thin, pectinase-sensitive outer layer. Protoplasts are formed by enzymatic digestion of some type-2 walls. The type-3 wall consists of a single homogeneous layer that resists degradation by polysaccharide-degrading enzymes (Yamada and Sakaguchi, 1982). *C. sorokiniana* was not examined in this study, but in a TEM photograph a *C. sorokiniana* cell appears to have a type-3 wall (Biedlingmaier et al., 1986).

Three additional cell wall characteristics, rigid wall polysaccharide, ruthenium red stainability, and anisotropy (a measure of degree of wall crystallinity) provide the most useful indicators to date of phylogenetic relationships in *Chlorella*. Species are divided into two groups based upon polysaccharide composition of the rigid portion of the wall. Rigid wall polysaccharides are composed either of glucose and mannose or glucosamine. The four strains of *C. sorokiniana* tested had a glucosamine rigid walls, positive anisotropy (indicating a crystalline wall), and could not be stained with ruthenium red. *C. sorokiniana* is the only species tested that has a glucosamine rigid wall and positive anisotropy (Takeda, 1988a, 1988b, 1991, 1993). Based upon both biochemical and cell wall characteristics, *C. sorokiniana* is most closely related to *C. vulgaris* and *C. kessleri* (Kessler and Huss, 1992; Takeda, 1993).

Chlorella Cell Wall Composition

There are several reports on monosaccharide composition of polysaccharides and protein concentration in *Chlorella* cell walls. Structure of wall polysaccharides, and whole wall, have not been studied. The only compound identified in any *Chlorella* wall other than carbohydrate or protein is sporopollenin (to be discussed in another section).

As in most cell walls, carbohydrate is the major cell wall constituent in *Chlorella*. As discussed briefly in a previous section, the *Chlorella* rigid wall consists of polymers of either glucose and mannose or glucosamine. Species with a glucose-mannose rigid wall have matrix polysaccharides rich in mannose and glucose and, in some cases, galactose and/or fucose. Species with glucosamine rigid walls have matrix polysaccharides rich in rhamnose and galactose and, in most cases, lesser amounts of fucose, xylose, arabinose, and glucose (Takeda, 1991). 3-*O*-Methyl-D-galactose was identified as a cell wall constituent of *C. vulgaris* K-22 (Ogawa et al., 1994).

C. sorokiniana and its relatives *C. vulgaris* and *C. kessleri* have, in order of decreasing concentration, rhamnose, xylose, mannose, galactose and glucose composing the matrix polysaccharides. In addition, *C. kessleri* has a relatively high percentage of fucose (Takeda, 1991, 1993). Three symbiotic strains of *Chlorella* also have cell wall compositions similar to the *C. sorokiniana* group (Kapaun et al., 1992).

The following are generalizations about the quantities of monosaccharide present in cell wall polysaccharides in *Chlorella* species (in % of wall dry weight). The *C. sorokiniana* wall was not analyzed in any of these studies. Three strains of *C. ellipsoidea* have 15-39% glucose in the alkaline-resistant wall fraction (Takeda and Hirokawa, 1978). Neutral sugars make up from 23%-44% of the cell wall polysaccharides of *Chlorella* species with a glucosamine rigid wall and up to 80% of the wall of those species with a glucose/mannose rigid wall. In these studies, neutral sugars were analyzed in H_2SO_4 hydrolysates. Some monosaccharides are unstable in H_2SO_4 resulting in a possible underestimation of neutral sugar concentration (Fry, 1988). For those species possessing a glucosamine rigid wall, 6.3%-15% of the wall contains glucosamine. For all species tested, uronic acid comprises 4.1%-24% of the wall (Northcote and Goulding, 1958; Loos and Meindl, 1982; Blumreisinger et al., 1983; Kapaun et al., 1992).

Protein concentration in *Chlorella* walls ranges from 1.7%-17% of the cell wall dry weight. Cell wall protein amino acid composition has been analyzed only for the alkaline-extracted cell wall residue of several strains of *C. ellipsoidea*. Since alkaline-extraction removes most cell wall protein, this report is not representative of the wall of a whole *Chlorella* cell. Hydroxyproline was present in one of four strains of *C. ellipsoidea* tested (Takeda and Hirokawa, 1984).

Enzymatic Degradation of Cell Walls

Autolytic Enzyme Activities

Cells protected by cell walls must have autolytic enzymes to degrade that wall to allow for cell expansion and division. The *Chlorella* mother wall is not completely digested during cell division, as evidenced by accumulation of empty walls in the medium of growing cultures. It is likely that a combination of precise enzymatic and mechanical mechanisms is involved in release of daughters from the surrounding mother wall. Enzymes with cell wall autolytic activities have been identified in several species of *Chlorella*. The study of these enzymes can provide valuable information about cell wall structure.

Enzymes that degrade polysaccharides into units of both high and low molecular weights and have pH optima from 5-6 are typical in *Chlorella* species having mannose and glucose in the rigid wall (Araki and Takeda, 1992). The endoenzymes β-1,4-mannanase, carboxymethyl cellulase, and β-D-fucosidase are associated with the *C. fusca* cell wall (Loos and Meindl, 1984; 1985). The endoenzymes selectively degrade wall polysaccharides into oligomers, which cytoplasmic enzymes degrade and use for daughter wall synthesis. The exoenzymes β-D-mannosidase, β-D-fucosidase, and β-D-glucosidase are found in supernatants of *C. fusca* cell extracts and degrade the mother wall (Walter and Aach, 1987; Araki and Takeda, 1992).

Cell wall lytic enzymes that degrade walls into mainly high molecular weight oligosaccharides and have pH optima around 8 are more typical for those species of *Chlorella* with a glucosamine rigid wall (Araki and Takeda, 1992). A protease was identified in *C. ellipsoidea* C-27 (reclassified as *C. vulgaris* 30.80) that cleaves peptide bonds of cell wall proteins (Takeda, 1991). Characterization of this enzyme activity as a protease is supported by its alkaline pH maximum and inhibition by a protease inhibitor (Hatano et al., 1992). Cell wall autolytic activity of *C. sorokiniana* 211-8k has a particularly broad pH optimum from 6-8. It is likely there are multiple enzymes, possibly protease(s) and polysaccharidease(s), responsible for the autolytic activity seen in this alga (Araki and Takeda, 1992).

Protoplast Production

Protoplasts have been produced from many types of cells of both monocot and dicot plants. There is no single procedure for preparation of protoplasts, but some generalizations can be made. Different tissues within a single plant and cells of different ages yield much different quantities of viable protoplasts. Growth conditions, such as the amount of light and nitrogen source, also have an effect on protoplast frequency.

In a typical procedure for the preparation of protoplasts, plant cells are treated for 2 to 24 h with cellulase, hemicellulase, and/or pectinase. An enzyme solution is buffered to pH 5.5 and contains 0.2-4% of each enzyme and an osmoticum. An osmoticum usually consists of 0.3-0.7 M mannitol, sorbitol, sucrose, or glucose (Evans and Bravo, 1983; Eriksson, 1985).

Protoplasts have been prepared successfully from several species of *Chlorella*. Incubation of *C. ellipsoidea* 211-1b and *C. saccharophila* 211-9a for 90 h in a mixture of cellulase, hemicellulase, and pectinase produced 20% and 80% protoplasts, respectively. Protoplasts were produced from cells only when they were treated with enzymes while being bubbled with air in the light (Braun and Aach, 1975). In another experiment, twelve *Chlorella* strains, including two strains of *C. sorokiniana,* were tested for the production of protoplasts. Of the twelve strains, only *C. ellipsoidea* C-87 and *C. saccharophila* 211-1a produced a large percentage of protoplasts. Protoplasts were produced within 24 h after treatment of cells with cellulase, Macerozyme (a pectinolytic enzyme, Patnaik and Cocking, 1982), and pectinase (Yamada and Sakaguchi, 1981).

Using cellulase, Macerozyme, and pectinase, a low percentage of protoplasts was made from *C. vulgaris* C-135 and C-169 (Yamada and Sakaguchi, 1982). Using cellulase alone, a higher percentage of protoplasts was prepared from an unidentified *C. vulgaris* strain from Carolina Biological (Berliner, 1977). Since publication of these data, many strains of *Chlorella* have been reclassified. Therefore, it is unclear if the strains of *C. vulgaris* tested have the glucosamine rigid wall typical of the species (Kessler and Huss, 1992).

Of those species of *Chlorella* known to have the glucosamine rigid wall, protoplasts have been produced only from *C. ellipsoidea* C-27 (reclassified as *C. vulgaris* 30.80). The treatment of *C. ellipsoidea* C-27 with a combination of a glycosidase mixture, chitosanase, and Macerozyme for 24 h resulted in 81% osmotically labile cells. Microscopic observation of osmotically labile cells revealed that they still retained an intact cell wall (Yamada et al., 1987). A homogenate of the *C. ellipsoidea* C-27 cells was prepared by breaking washed cells in a French pressure cell, removing cell debris by centrifugation, and concentrating the supernatant. The supernatant contained a lytic enzyme, as demonstrated by its ability to solubilize up to 30% of the [^{14}C]-labeled cell wall as a high molecular weight polysaccharide. The lytic enzyme has a pH optimum of 8. This enzyme was a protease that cleaved peptide bonds of proteins that bridged cell wall polysaccharides. Chitosanase from *Bacillus* R-4 partially solubilized the wall at pH 5.6 and 7.6. The chitosanase, as prepared, is known to have a contaminating protease which may be responsible for the cell wall lytic activity. The cell wall was also partially solubilized by treatment with Pronase P or trypsin (Satoh and Takeda, 1989). The [^{14}C] was not released from the wall when treated with other polysaccharide-degrading enzymes.

Protoplasts were ultimately produced from *C. ellipsoidea* C-27 using mixed glucanases, chitosanase from *Bacillus* R-4, and algal cell homogenate. Each of the three enzyme components alone produced less than 12% osmotically labile cells. Chitosanase plus homogenate produced 67% osmotically labile cells. In a mixture of all three enzyme components, approximately 90% of the cells were osmotically labile within 3 h at pH 7. Glucanases, chitosanase, and homogenate were all essential for efficient protoplast formation. Greatest protoplast frequency was obtained from cells harvested at least half way through the cell cycle. The protease inhibitor PMSF decreased protoplast frequency by 70%. Absence of a cell wall was confirmed using TEM (Hatano et al., 1992).

Cell Wall Factors that Decrease Enzymatic Wall Digestion

Lignin and Lignin-Like Compounds

Lignin and lignin-like compounds, such as phenolic acids and other phenolic biopolymers, are very common in walls of higher plants and occur only rarely in algal walls. The presence of lignin and some phenolic compounds correlates with poor wall digestibility. The mechanism by which lignin prevents wall digestion is not completely clear. Lignin probably prevents access of enzymes to other cell wall substrates by decreasing cell wall pore size or adsorbing enzymes (Hartley et al., 1990; Jung et al., 1992; Converse 1993, Besle et al., 1994). Several methods of chemical delignification of forage stems, in some but not all cases, resulted in an increase in digestibility of substrate by rumen microorganisms. It is possible that disruption of the wall and not delignification contributed to increased digestion (Jung et al., 1992). An increase in lignin content in sorghum cell walls as the plants aged was paralleled by an increase in *p*-coumaric acid and a decrease in cellulose degradability. It was proposed that an increased association of phenolic compounds with cellulose is responsible for the decrease in cellulose degradability (Goto et al., 1991). High *p*-coumaric acid also correlated with low digestibility of pearl millet (Hartley et al., 1992).

Resistance of two algal cell walls to microbial digestion led to the discovery of lignin-like compounds. The wall of *Staurastrum* sp. was fractionated, and fractions were subjected to microbial degradation. A fraction that was not degraded by a mixed microbial culture contained a lignin-like compound (Gunnison et al., 1975). Resistance of the *Coleochaete* wall to microbial degradation is also believed to result from the presence of a phenolic biopolymer (Delwiche et al., 1989).

Sporopollenin

Sporopollenin was first described as a highly resistant compound present in outer layers of pollen and fungal spores. It has since been found in cell walls of many green algae (Atkinson et al., 1972; Brunner and Honegger, 1985). Because of resistance of sporopollenin to chemical solubilization and enzymatic degradation, the chemical structure has not yet been elucidated. Sporopollenin is defined based upon its solubility characteristics. It is insoluble in most acids, bases, lipid solvents, and detergents. It is also resistant to acetolysis (boiling in 9:1 acetic anhydride and concentrated sulfuric acid). Lignin, carotenoids, and 6-deoxysugars, such as rhamnose and fucose are among the few other organic compounds that are also resistant to acetolysis (Southworth, 1974; Brunner and Honegger, 1985; Fry, 1986).

Sporopollenin was first proposed to be a polymer of carotenoids and/or carotenoid esters. Plants and fungi grown with [^{14}C]-acetate, [^{14}C]-mevalonate (carotenoid precursors), or [^{14}C]-β-carotene incorporated [^{14}C] into pollen and spore sporopollenin. Sporopollenin has some chemical characteristics in common with carotenoids such as pyrolysis-gas chromatograms, elemental analysis, and x-ray diffraction patterns. Also like carotenoids, sporopollenin absorbs UV light and is autofluorescent (Brooks and Shaw, 1978; Singh and Devi, 1992).

The "three-way correlation" (Atkinson et al., 1972) predicts that those cells, which have a cell wall with an outer trilaminar layer and produce ketocarotenoids, also have sporopollenin. This hypothesis is supported by mutant studies on *Chlorella fusca*, an alga whose phenotype is consistent with the "three-way correlation." Mutants were selected based upon carotenoid deficiency. All mutants lacked a trilaminar layer and sporopollenin (Burczyk and Hesse, 1981; Burczyk and Czygan, 1983). The parasitic alga *Prototheca*, possibly an apochlorotic form of *Chlorella,* does not fit the "three way correlation." *Prototheca* has an outer trilaminar wall layer and sporopollenin, but lacks carotenoid pigments and the colorless carotenoid precursors phytoene and phytofluene (Puel et al., 1987).

Conflicting results have been obtained from studies on effects of carotenoid inhibition on sporopollenin accumulation. The bleaching herbicide norflurazon (also called Sandoz and SAN 9789) competitively inhibits phytoene desaturase, the enzyme of the first committed step of carotenoid biosynthesis (Sandmann et al., 1991). Norflurazon-treated cultures of the green alga *Scenedesmus obliquus* had a 87% decrease in ketocarotenoid content and a 50% decrease in sporopollenin concentration (in cell wall dry weight) (Burczyk, 1987). Pollen extracts from squash plants (*Cucurbita pepo*) treated with norflurazon had decreased carotenoid concentration and accumulation of the nonpigmented carotenoid precursors phytoene and phytofluene. The decrease in carotenoid concentration was not reflected by a decrease in sporopollenin concentration (Prahl et al., 1985, 1986).

Inhibition of sporopollenin synthesis and termination of pollen development was observed in plants treated with MON-20763 (also called RH-0007 and fenridazon), a phenyl pyridazone (Cross and Ladyman, 1991). Two phenyl-cinnoline carboxylate compounds, SC-1058 and SC-1271, have similar chemical structures and effects on pollen development as does MON-20763. Abnormal vacuolation, possibly a result of coalescence of secretory vesicles, was observed in SC-1058- and SC-1271-treated pollen. El-Ghazaly and Jensen (1990) proposed that MON-20763 interferes with pollen development by preventing polymerization of sporopollenin monomers. Other workers proposed that the mechanism of developmental inhibition of pollen for all three compounds is the interference of transport of sporopollenin monomers from the cytoplasm to the cell wall (Schulz et al., 1993). These compounds have not been tested on sporopollenin synthesis in lower eucaryotes.

Solid state [^{13}C] NMR spectroscopy results showed that sporopollenin from algae (including *C. fusca*) and higher plants do not have structural characteristics of carotenoid polymers. Sporopollenin is a polymethylenic carbon chain with a high degree of saturation. Sporopollenin can vary in the numbers and types of oxygenated compounds such as ether, hydroxyl, ketone, ester and carboxylic acids. Sporopollenins should be considered as a group of related compounds (Guilford et al., 1988; Espelie et al., 1989; Derenne et al., 1992).

Pyrolysis GC-MS of sporopollenin from *Chlorella fusca* and *Nanochlorum eucaryotum* did not detect isoprenoid carbons. Polymethylenic chains of the sporopollenin from these algae had up to 30 carbons (Derenne et al., 1992). Using pyrolysis mass spectroscopy of sporopollenin from *Pinus mugo* pollen, *p*-coumaric acid was detected. It is likely that *p*-coumaric acid is a structural unit in some sporopollenins, and may contribute to sporopollenin autofluorescence (Wehling et al., 1989).

Cell Wall Structure

Composition and structure of cell wall polysaccharides can contribute to resistance of the wall to enzymatic digestion. Crystalline cell wall polysaccharides are more resistant to degradation than are amorphous matrix polysaccharides. Strong negative correlation between degree of crystallinity and rate of wall hydrolysis has been demonstrated. Incomplete enzymatic hydrolysis of highly crystalline substrates is often due to low surface area of the substrate (Converse, 1993).

Pectins vary in their sensitivity to pectinases. Branched or 'hairy' pectins are often resistant to pectinase whereas unbranched or 'smooth' pectins are more pectinase sensitive (Fry, 1988). A heteropolymer containing galacturonic acid in the wall of the alga *Fischerella muscicola* is probably the component rendering this wall highly resistant to microbial decomposition (Gunnison and Alexander, 1975).

MATERIALS AND METHODS

Culture Conditions

Autotrophic growth of *Chlorella* cells was performed in light in SUN medium as described by Prunkard et al. (1986). This basal salts medium contained in mM concentration: KNO_3, 29.0; $CaCl_2$, 0.340; K_2SO_4, 6.00; KH_2PO_4, 18.4; $MgCl_2$, 1.50; in µM concentration $CoCl_2$, 0.189; $CuCl_2$, 0.352; EDTA, 72.0; $FeCl_3$, 71.6; H_3BO_3, 38.8; $MnCl_2$, 10.1; NH_4VO_4, 0.200; $(NH_4)_6Mo_7O_{24}$, 4.19; $NiCl_2$, 0.190; $SnCl_2$, 0.190; $ZnCl_2$, 0.734. After autoclaving, medium was adjusted to pH 6.8 with 0.5 N KOH. *C. sorokiniana* cells were cultured in a 38.5°C water bath with mirrors behind fluorescent lamps. *C. fusca, C. ellipsoidea*, and *C. saccharophila* were grown in a 25°C lighted water bath without mirrors. All cultures were bubbled with a 2% (v/v) CO_2-air mixture.

For heterotrophic growth, *C. sorokiniana* cells were cultured in SUN medium supplemented with 50 mM glucose. Cultures were grown at 37°C in 1 L Erlenmeyer flasks in darkness while shaking at 200 rpm.

Cultures were maintained in the light at approximately 22°C on slants of SUN medium supplemented with 1.5% agar.

Source of *Chlorella* Cultures

C. sorokiniana strain IIIB2NA #7 was derived from Dr. C. Sorokin's original environmental isolate from warm surface waters in Austin, Texas. It has been maintained in the laboratory under autotrophic conditions on agar media for about 38 years. *C. sorokiniana* strain 211-8k is also derived from Sorokin's original culture (Starr and Zeikus, 1993). Prior to initiation of experiments presented herein, the *C. sorokiniana* culture was streaked for isolation on agar medium. All experiments were conducted on cultures derived from one colony.

C. ellipsoidea C-87 and *C. saccharophila* C-211 were obtained from the culture collection at the Institute of Applied Microbiology at the University of Tokyo. *C. fusca* was obtained from the University of Texas culture collection of algae.

Preparation of Samples for Transmission Electron Microscopy

Samples were harvested by centrifugation and fixed for 1 h at room temperature in microfuge tubes containing 2% (v/v) acrolein (Sigma) and 2% (v/v) glutaraldehyde (Tousimis) in 0.2 M cacodylate buffer (Sigma), pH 6.8. After centrifugation and removal of supernatant, pellets were washed 2-times in cacodylate buffer and incubated in a 1% solution of osmium tetroxide (Pella) in cacodylate buffer for either 1 h at room temperature or overnight at 4°C. Pellets were washed 2-times with water and dehydrated sequentially for 10 min in each of 5 solutions containing 25%, 50%, 75%, 95%, and 100% ethanol followed by 15 and 30 min in acetone. Dehydrated sample pellets were embedded at room temperature in Spurr's Low Viscosity Resin by incubating pellets in 30% plastic in acetone for 1 h followed by 70% plastic for 1 h and 100% plastic for 2 h. Cells in 100% plastic were incubated at 60°C for approximately 12 h or until hardened.

Thin sections of embedded samples were cut on a microtome (RMC) and placed on formvar-coated copper grids. Thin sections were post-stained with freshly filtered 2% $KMnO_4$ for 5 min, rinsed with water, bleached for 30 s with a solution containing 3 drops of 1.2% sodium sulfite and 3 drops of 1% oxalic acid in 10 mL of water, rinsed with water, stained with Reynold's lead citrate for 30-60 s, and rinsed with water (Atkinson, 1972). Samples were observed using a Zeiss electron microscope.

Preparation of Samples for Scanning Electron Microscopy

Samples for SEM were fixed in the same manner as those for TEM. After dehydration of samples in 100% ethanol, samples were placed in HMDS for 20 min, centrifuged, and HMDS was removed and samples were air dried. Dry samples were sputter-coated with gold before observation on a Hitachi S-4000 scanning electron microscope.

Determination of Cell Wall Thickness

Images from 3.25" x 3.9" negatives were enlarged to 8" x 11" and printed on photographic paper. Cell wall image widths in mm were measured. To determine actual wall thickness, width of the wall obtained from each photograph was divided by magnification of the microscope and also by amount enlargement from negative to print (2.5 times for an 8" x 11" print). The final value was the cell wall thickness in micrometers. Reported cell wall thickness resulted from calculations compiled from at least ten photographs.

Purification of *Chlorella* Cell Walls

Cell walls were purified from cells cultured heterotrophically in darkness for the following experiments: carbohydrate, protein, and amino acid analyses; NMR analysis; acetolysis of walls from norflurazon-treated cells. For remaining experiments, walls were purified from cells cultured autotrophically in light. For all wall purifications approximately 5 L of logarithmically growing *Chlorella* cells were centrifuged at 20,000*g* for 10 min at 4°C. Cell pellets were washed once with dH_2O and stored at -20°C until needed for cell wall isolation. Breaking, extraction, and centrifugation of *Chlorella* cells were performed at 4°C unless stated otherwise. Thawed pellets were diluted with approximately 3 volumes of dH_2O and broken 3-times in a French press at 20,000 p.s.i. The soluble cell fraction was removed by centrifugation in 30 mL Corex centrifuge tubes at 20,000*g* and pellets were washed with water repeatedly until the supernatant was colorless. After each centrifugation step, pellets were suspended in water leaving behind a dense, white, starch containing portion of the pellet. Remaining starch or whole cells were removed by centrifugation 3-times at 1,000*g* for 15 min. Removal of whole cells and starch was confirmed microscopically. Partially purified walls were extracted in acetone until the supernatant was colorless. Four additional extractions were performed for 10 min at 50°C before centrifugation; two in acetone and two in methanol. Purified walls were suspended in dH_2O and lyophilized over night (Brunner and Honegger, 1985). Cell walls purified for carbohydrate analysis were treated with amylase (Sigma).

Dry Weight Determinations

Prior to weighing, aluminum pans were dried in an oven at 100°C for at least 24 h. Preweighed pans containing samples were incubated at 100°C for 24 h and weighed again. Sample dry weights were determined by subtracting dry weight of a pan from the dry weight of pan and sample.

Instruments used for Spectroscopic Analyses

Infrared spectrometry was performed using a Perkin Elmer 1600 Series FTIR spectrometer at the University of Florida, Department of Chemistry, Spectroscopic Services Laboratory. GC-MS of carbohydrates was performed at the University of Florida, ICBR, Glycobiology Core Laboratory on a Shimadzu QP-5000 GC-MS work station. Amino acid analyses were performed at the University of Florida, ICBR, Protein Core Laboratory on a Beckman 6300 Amino Acid Analyzer. Elemental analysis was performed at the University of Florida, Department of Chemistry, Spectroscopic Services on a Fisons 1108 CHN Elemental Analyzer.

Extraction of Hydrolyzable Carbohydrates from *Chlorella* Walls

Acid Hydrolysis of Cell Walls

Hydrolysis of cell wall samples for GC-MS analyses was performed at the Glycobiology Core Laboratory by laboratory staff. Hydrolysates used in spectrophotometric assays were prepared by the author using different chemical stocks and different facilities than those at the Glycobiology Core.

Heating of cell wall samples in acid solutions was conducted under nitrogen gas in 3 or 5 mL thick-walled V-vials (Wheaton). Samples were hydrolyzed at 100°C and 110°C in a heating block or an autoclave. TFA hydrolysates were prepared by incubating 2-10 mg of cell walls in 1-2 mL of 2 M TFA at 100°C for 6 h. After removal of the unhydrolyzed wall by centrifugation, supernatant containing the TFA hydrolysate, was evaporated under a stream of nitrogen gas or in a Speed Vac Concentrator (Savant). H_2SO_4 hydrolysates were prepared by soaking residue from TFA hydrolysis in 0.5 mL of 72% (w/v) for 4 h at room temperature. The H_2SO_4 was diluted to 4% with dH_2O and further hydrolysis was performed at 100°C for 4 h. The supernatant, present after centrifugation of the H_2SO_4 treated walls, was designated the H_2SO_4 hydrolysate. The H_2SO_4 hydrolysate was neutralized with solid barium carbonate. After centrifugation at 15,000*g* to remove solid barium carbonate, the supernatant was dried in a rotary evaporator. In another sample, residue remaining after TFA hydrolysis was hydrolyzed further in 1-2 mL of 6 N HCl for 18 h at 110°C. Unhydrolyzed cell wall residue was removed by centrifugation and HCl was evaporated under a stream of nitrogen or in a Speed-Vac. Residues remaining after each acid hydrolysis procedure were dissolved in dH_2O prior to analysis (Takeda, 1991).

Monosaccharide composition of TFA and HCl hydrolysates was determined using GC-MS. Prior to gas chromatography, monosaccharides were converted to their tetramethylsilane (TMS) derivatives and amino sugars were acetylated. Monosaccharides were identified based upon retention time (gas chromatography) and mass to charge ratio (mass spectrometry). An external standard consisted of known molar concentrations of inositol and each monosaccharide in the hydrolysate. For each monosaccharide, a response factor (ratio of peak area to mol of monosaccharide) relative to inositol, was determined. Monosaccharide concentrations were calculated using the response factor.

Hydrolysates were also analyzed using spectrophotometric assays. TFA hydrolysates were analyzed for uronic acids using the metahydroxy diphenyl assay (Chaplin and Kennedy, 1986). H_2SO_4 and HCl hydrolysates were analyzed using the phenol sulfuric assay for total carbohydrate, the Elson-Morgan assay for amino sugars (Chaplin and Kennedy, 1986), and the glucose oxidase assay (Sigma, Procedure No. 510).

Alkaline Hydrolysis of Cell Walls

Alkaline hydrolysis was performed on purified *C. sorokiniana* cell walls in both 0.4 N and 1 N NaOH. For a 0.4 N NaOH hydrolysis, 5.0 mg of walls were incubated in 1.5 mL of NaOH solution under nitrogen for 20 h at 30°C (Takeda and Hirokawa, 1978). For a 1 N NaOH hydrolysis, 0.26 g of walls were extracted with 9 mL of NaOH solution under nitrogen 2-times for 20 h each at 30°C (Morrison et al., 1993). Hydrolysate was recovered by precipitation in 2 volumes of ethanol at -20°C over night. Hydrolysates were dried under nitrogen gas prior to resuspending in dH_2O. Alkaline-soluble cell wall fraction was analyzed using proton-decoupled [^{13}C] NMR spectroscopy. Alkaline-insoluble wall residue was analyzed using IR spectrometry and elemental analysis.

Protein and Amino Acid Analyses of Purified Cell Walls

Cell wall protein was extracted from approximately 15 mg of purified cell walls in 1 mL of 1 N NaOH. Samples were incubated while shaking at 37°C for 20 h (Loos and Meindl, 1982). Extracts were analyzed using the BioRad and Lowry et al. (1951) protein assays.

Amino acids were extracted from cell walls by incubating approximately 20 mg of walls in 1.7 mL of 6 N HCl containing 1% phenol and 0.02% mercaptoethanol at 110°C for 20 h (Loos and Meindl, 1982). Supernatants were recovered by centrifugation in a microfuge and evaporated using a Speed Vac. Residues were solubilized in dH_2O and subjected to automated analysis of amino acid composition. Total wall protein was calculated from amino acid composition data. An average amino acid molecular weight of 125 was calculated based upon mol% of constituent amino acids. The final value was corrected for weight of a water molecule lost upon formation of a peptide bond between each of two amino acids in the peptide.

Acetolysis

Whole cells, purified cell walls, chitin (Sigma), and chitosan (Sigma) were subjected to acetolysis as described by Brunner and Honegger (1985). All steps were performed at room temperature unless stated otherwise. After each step, samples were centrifuged in 30 mL Corex centrifuge tubes at

20,000*g* at 4°C. Prior to acetolysis, purified cell walls were washed sequentially in methanol for 5 min at 50°C, 1:2 methanol:chloroform for 5 min at 50°C, and in 1 N NaOH and acetic acid each for 10 min. Acetolysis was performed by suspending washed walls in 1:9 (v/v) concentrated sulfuric acid:acetic anhydride and placing tubes in a boiling water bath for up to 30 min. Residues were washed sequentially in acetic acid, 0.1 M sodium acetate, 0.025 M phosphate buffer (pH 7), dH_2O, and methanol and dried in a 60°C oven.

TFA treatment of cell walls prior to acetolysis was performed by heating approximately 50 mg of walls in 1.5 mL of 1 M TFA in a 3 mL V-vial to 100°C for 1 h (Fry, 1988). Residues were washed with dH_2O and subjected to acetolysis as described above. Alkaline extraction, prior to acetolysis, was performed on approximately 50 mg of dried walls by successive extraction in 10% (v/w) aqueous KOH for 5 h at 100°C, 5% (v/w) ethanolic KOH (95% ethanol) for 3 h at 75°C, dH_2O 10 min at 100°C, and dH_2O until neutralization (Burczyk, 1987).

Assessment of Cell Wall Autofluorescence

Autofluorescence of purified substrates was determined by observing a wet mount with a Nikon Labphot fluorescence microscope. Samples were observed for yellow fluorescence upon excitation with blue light (Singh and Devi, 1992).

Determination of Cell Wall Solubility in Phosphoric Acid

Approximately 80 mg of dried, alkaline-extracted (in 10% (w/v) KOH as described above) walls were incubated in 20 mL of 85% phosphoric acid in a 55°C water bath for up to 30 d. Prior to dry weight determination, insoluble residues were washed with dH_2O until a neutral pH was obtained, followed by washing with methanol 3-times at 65°C, acetone 3-times at room temperature, and once with ethyl ether (Burczyk, 1987).

Phloroglucinol Assay for Lignin

Using the phloroglucinol assay (Berkaloff et al., 1983), purified *C. sorokiniana*, *C. fusca*, and *C. ellipsoidea* cell walls were tested for lignin content. Pine saw dust was used as a positive control. A drop of a saturated phloroglucinol (Sigma) solution, prepared in 20% HCl (v/v), was placed on a sample of saw dust or cell walls on a glass slide. A positive reaction for lignin was indicated by formation of a magenta color.

Analysis of Alkaline Extracts for the Presence of Phenolic Compounds

Approximately 0.25 g of purified *C. sorokiniana* cell walls was incubated in two 9 mL aliquots of 1 N NaOH under nitrogen each for 20 h at 25°C. Alkaline insoluble wall residue was removed by centrifugation. The combined supernatants were adjusted to pH 1 with 2 M TFA and extracted

3-times with 1-butanol (Morrison et al., 1993). A UV spectrum was taken of combined butanol extracts (Beckman, model DU 640). Butanol was evaporated under a stream of nitrogen. Residue was dissolved in 5 mL of water before analysis by proton-decoupled [^{13}C]-NMR spectroscopy.

Determination of Cell Wall Solubility in Chromium Trioxide

Approximately 25 mg of purified walls were incubated in a solution containing 2 g CrO_3 in 2 mL of dH_2O and 2 mL of acetic acid. Solubility of cell walls in the CrO_3 solution was assessed within 7 d incubation at room temperature (Berkaloff et al., 1983).

Preparation of Cell Wall Degrading Enzymes

Enzymes Tested

Enzymes tested to degrade purified walls and walls of intact cells were: 2% (w/v) Cellulysin (Calbiochem), 2% (w/v) hemicellulase (Sigma), 1% (w/v) pectinase (Sigma) (each singly and in a mixture of the three enzymes referred to as CHP), a mixture of 0.4 U/mL chitinase (Sigma), 1.9 U/mL N-acetylglucosaminidase (Sigma), 1% (w/v) lysozyme (Sigma), and 0.2% Pronase (Sigma). Chitosanase, purified from *Streptomyces lividans* pRL207, and a crude *C. sorokiniana* cell homogenate were also tested. Enzymes tested only for their ability to produce osmotically labile cells were cytolase

123, hemicellulase HP 150, cytolase 300 (Genecore), pectolyase Y23, cellulase RS, and Macerozyme R10 (Karlan Research Products).

Growth of *S. lividans* pRL207 for Chitosanase Production

A *S. lividans* culture was started by transferring a generous amount of culture from a slant (provided by R. Brzezinski) into a 125 mL Erlenmeyer flask containing 30 mL of TSB (Gibco) plus 50 µg/mL of filter-sterilized kanamycin. The TSB culture was incubated at 30°C while shaking at 250 rpm until dense growth was observed (5-7 d). Sporulation agar plates (SLM3 medium) (DeWitt, 1985) were inoculated with 0.5 mL of TSB culture. Plates were incubated at 30°C for about 10 d. Spores grown on SLM3 medium served as inoculum for precultures. SLM3 agar contained in g/L: starch, 10.0; corn steep liquor, 5.00; $CaCO_3$, 3.00; $FeSO_4 \cdot 7H_2O$, 0.022; agar, 20.0. pH was adjusted to 5.8 with HCl and medium was sterilized by autoclaving.

The preculture was grown in 50 mL of TSB plus 50 µg/mL kanamycin in a 250 mL Erlenmeyer flask. TSB was inoculated heavily with gray spores that formed on the surface of an SLM3 plate. The culture was incubated for 48 h at 30°C while shaking.

The preculture was centrifuged in a sterile 15 mL polypropylene tube. The supernatant was discarded. Bacterial pellet volume was estimated and cells were suspended in two pellet volumes of sterile 0.9% NaCl. One hundred milliliters of chitosanase-production medium (in a 1 L Erlenmeyer flask) were inoculated with 2 mL of bacterial suspension. Chitosanase-production medium contained in g/L: K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 2.0; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; chitosan (milled in a coffee-bean grinder), 15.0; starch, 5.0 and in mL: olive oil, 2.0. pH was adjusted to 7 with HCl and the medium was sterilized by autoclaving. After sterilization, the following filter-sterilized components were added to 1 L of medium: 100 mL 1 M MOPS buffer, pH 7; 1 mL trace elements solution (which contained in mg/L: $ZnCl_2$, 40; $CuCl_2$, 10; boric acid, 10; $(NH_4)_6Mo_7O_{24}$, 10), and 1 mL 10 mg/mL riboflavin. The culture was incubated for 72 h at 30°C while shaking at 250 rpm. Culture supernatant was used as starting material for chitosanase purification (Masson et al., 1993; Boucher and Brzezinski, personal communication).

Purification of Chitosanase

Supernatant from the chitosanase production medium was harvested by centrifugation at 4°C. EDTA was added at a final concentration of 2.5 mM. The supernatant was acidified to pH 4.5 with 5 M acetic acid. A 2% solution of polyacrylic acid (M_r 250,000, Aldrich Chemical Co.) was added dropwise to the supernatant until polyacrylic acid concentration was 4-times higher than protein concentration (Stoscheck, 1990). The solution was stirred for 30 min and a precipitate was recovered by centrifugation. Precipitate was suspended in

300 mL of dH_2O and brought to pH 8.5 with 1 M NaOH. Residual polyacrylic acid was precipitated by addition of 1 M calcium acetate dropwise to a final concentration of 35 mM. Precipitate was removed by centrifugation and discarded. The supernatant, which contained chitosanase, was stored at 4°C or diluted with an equal volume of sterile 20% glycerol and stored at -20°C.

All centrifugation steps were performed at 11,000*g* for 15 min. A temperature of 4°C was maintained throughout the purification procedure.

Chitosanase Assay

Chitosanase activity was tested on soluble chitosan (solubilized as a 10 mg/mL stock in 1 N HCl) and insoluble chitosan which were diluted to prepare a 0.1% chitosan solution (v/v) or suspension (w/v) in 50 mM sodium acetate, pH 5.5. Assay mixtures of 950 µL of chitosan solution and 20 mU of chitosanase were incubated for 10 min at 37°C (Boucher et al., 1992). The supernatants were assayed for reducing sugars using the neocuproine assay (Chaplin and Kennedy, 1986). To prevent precipitation of samples prior to reading on the spectrophotometer, they were maintained at 37°C.

Production of *C. sorokiniana* Cell Homogenate for Cell Wall Degradation

Three 800 mL cultures of *C. sorokiniana* cells were synchronized by subjecting cells to a 10:7:9.5 h light:dark:light cycle. The starting culture had an absorbance (640 nm) of 1. After the dark period, the cultures were again diluted to an absorbance (640 nm) of 1. At the end of the final light period (i.e., 9.5 h), cells were washed in water, suspended in water, and broken during one pass through a French pressure cell. To remove cell debris, homogenate was centrifuged at 4°C and 20,000*g* rpm for 30 min. Supernatants were lyophilized and stored at -20°C (Hatano et al., 1992). Protein concentration of the dried preparation was determined (Bradford, 1976). Immediately before use, dried supernatant was prepared as a 1.5% solution (w/v) in buffer resulting in a final concentration of approximately 5 mg protein/mL.

Treatment of Whole Cells with Cell Wall Degrading Enzymes

Chlorella cells were grown autotrophically in light, harvested during logarithmic growth, and placed in an Erlenmeyer flask overnight under light to allow for division of most cells into daughter cells. Before cells were harvested for treatment with cell wall degrading enzymes, the culture was grown autotrophically for an additional 2-4 h.

For treatment of cells with polysaccharide-degrading enzymes, cells were handled aseptically and all buffers and enzymes were filter sterilized. *Chlorella* cells were centrifuged in a clinical centrifuge. Pellets were washed once in buffer containing citrate, 0.1 M; EDTA, 60 mM; sorbitol, 0.5 M, pH 5.5. Pelleted cells were resuspended in the same buffer and counted using a hemocytometer. Cells were centrifuged in microfuge tubes and resuspended in buffer or buffer plus enzymes for a final volume of 5 mL of 4×10^7 cells/mL in 50 mL Erlenmeyer flasks. Flasks were incubated in the dark at 35°C for up to 16 h while shaking at 75 rpm. Enzyme-free controls were included with each experiment.

For experiments in which a protease and/or cell homogenate in conjunction with polysaccharide-degrading enzymes were tested for their ability to induce formation of protoplasts, the following conditions were applied. Daughter cells were cultured autotrophically in light for 2-4 h. Approximately 2.5×10^9 cells/mL were suspended in reaction buffer (25 mM sodium phosphate and 0.5 M mannitol, pH 6 or 7). Enzyme solutions prepared in reaction buffer contained all or a subset of CHP, Pronase, chitosanase, and homogenate. A solution containing 1 part cells, 8.5 parts enzyme mixture, and 0.5 parts 240 mM $CaCl_2$ were incubated in 1.5 mL microfuge tubes at 30°C, while shaking in the dark (Hatano et al., 1992).

After at least 4 h of enzyme treatment, formation of osmotically-labile cells was assessed. Cell lysis in a hypotonic solution was indicative of either a weakening or complete removal of the cell wall. Water was drawn under the coverslip of a wet mount of *Chlorella* cells. Osmotically-labile cells swelled and ruptured from this treatment. A visual estimation of percentage of broken cells was made.

Using ultrafiltration in a stirred cell with a Diaflow PM-10 filter (10,000 mwco, Amicon), reducing sugars were removed from commercial enzyme preparations Enzymes were diluted to working concentrations in 0.1 M citrate and 60 mM EDTA, pH 5.5. Solutions were concentrated at 37 psi to approximately 10% of the original sample volume. Buffer was added to return the concentrated sample to its original volume. This procedure was repeated a total of 3-times. The final concentrate was returned to the original volume with buffer (R. Brown, personal communication).

Treatment of Purified Substrates with Cell Wall Degrading Enzymes

Ten milligrams of cell walls were incubated at 30°C for 16 h while shaking at 75 rpm in 0.1 M citrate buffer adjusted to an appropriate pH for each enzyme. Supernatants were assayed for reducing sugars (dinitrosalicylic acid assay), total carbohydrate (phenol sulfuric acid assay), amino sugars (Elson Morgan assay), uronic acids (metahydroxy diphenyl assay) (Chaplin and Kennedy, 1986), and glucose (glucose oxidase assay, Sigma. Procedure No. 510).

The fluorescamine assay (Udenfriend et al., 1972) was used to assess enzymatic release of primary amines (amino sugars, amino acids, and peptides) from purified substrates. Substrates tested as positive controls were purified cell walls 0.1% (w/v), 0.1% (w/v) chitosan (both soluble and insoluble), 0.02% (w/v) BSA, and 0.65 µg-20 µg glucosamine.

In each reaction mixture, 950 µL of substrate in 25 mM sodium phosphate buffer, pH 6 or pH 7 were mixed with 50 µL of enzyme and dH_2O and incubated at 37°C for 1 h. Three-hundred microliters of supernatant diluted in 0.2 M sodium borate pH 7 were mixed with 100 µL of 0.01% (v/v) fluorescamine (Sigma) in acetone. Using a Shimadzu DR-15 fluorimeter with an excitation wavelength of 390 nm and an emission wavelength of 475 nm, fluorescence emission was determined.

Treatment of Thin Sections with Polysaccharide-Degrading Enzymes

Logarithmically growing *C. sorokiniana* and *C. fusca* cells were prepared for TEM as described previously except osmium was omitted and thin sections were placed on formvar-coated nickel grids. Before post-staining, grids were incubated on drops of CHP or a mixture of CHP, 1.5% w/v homogenate, 1.32 U pronase, and 20 mU chitosanase for 4 h at room temperature. Prior to microscopic observation, grids were washed with water and post-stained as described in a previous section.

Attempts to Induce Accumulation of Ketocarotenoids in *C. sorokiniana* and *C. fusca* Cells

C. sorokiniana and *C. fusca* cells were cultured autotrophically in light in SUN medium with KNO_3 concentration decreased to 0.08 mM. Cultures were incubated for up to 7 d. Accumulation of ketocarotenoids was confirmed using TLC (Kessler, 1978). Canthaxanthin (Fluka) and ß-carotene (Sigma) were used as standards.

Determination of Effects of Norflurazon on Growth Rate, Carotenoid Content, and Percentage of Acetolysis Resistant Cell Wall Fraction of *C. sorokiniana*

Norflurazon was provided by Robert Lamoreaux, Sandoz Agro, Inc.. To determine concentration of norflurazon required to decrease carotenoid content, while also having minimal affect on growth rate, *C. sorokiniana* cultures were grown heterotrophically in medium supplemented with several different concentrations of norflurazon. For carotenoid and wall analyses, cells were grown in 1 µM norflurazon for 3 doublings. Total carotenoid content of cells was determined (Liaaen-Jensen and Jensen, 1971). For identification of individual carotenoids, pigments were separated using TLC (Kessler, 1978). Pigmented bands from TLC plates were dissolved in both ethanol and chloroform. Using a Beckman DU 640 spectrophotometer, absorption maxima were measured in the two solvents and compared to published data (Britton, 1985).

Dry weight of acetolysis-resistant residues of whole *Chlorella* cells was determined (methods described in previous sections).

Determination of the Effect of Growth in MON-20763 on *Chlorella* Cells

MON-20763 was supplied by Dennis Dunphy, HybriTech Seed International, Inc. Cells were cultured autotrophically in the light in SUN medium supplemented with various concentrations of MON-20763. Cells were grown in cultures supplemented with the highest concentration of MON-20763 that would allow for the OD_{640} of the culture to double twice. Before and after treatment with CHP, *C. fusca* and *C. sorokiniana* cells, cultured in MON-20763, were observed under light microscopy and TEM. Whole cells were also subjected to acetolysis.

Production and Screening of Cell Wall Defective Mutants

Logarithmically growing *C. sorokiniana* cells were transferred to an Erlenmeyer flask and left in low light without agitation for 48 h. This treatment allowed most of the cells to divide into daughter cells, and to decrease their starch content. Cells were counted using a hemocytometer.

To construct a kill curve, *Chlorella* cells were centrifuged in a 50 mL polypropylene centrifuge tube and resuspended with SUN medium to result in 1.5×10^9 cells/mL. Twenty milliliters of cell suspension were transferred to a sterile, glass, 9 cm Petri dish. The Petri dish was placed in the center of a stir plate whose surface was 9" from the bulb of a UV lamp (Model MR-4, George Gates Co., Inc., with a Silvania G8T5, 8W bulb). Upon exposure to UV light, cells in the Petri plate were stirred as slowly as possible with 7 cm stir bar made by heat sealing a straightened paper clip inside a piece of Tygon tubing. To prevent induction of photo-repair mechanisms, efforts were made to minimize exposure of mutagenized cells to ambient light. At 1 min intervals (there was no UV exposure at zero time), for a total of 12 min, 100 µL of cell suspension were removed from the Petri dish and diluted in SUN medium to a final concentration of 1.8×10^3 cells/mL. A 200 µL aliquot of this final dilution (360 cells) was spread onto each of four SUN plus 50 mM glucose plates (1.5% agar) and incubated in the dark at 37°C. After a one week incubation, colonies on each plate were counted and plating efficiency and percent kill were calculated.

For production and screening of *C. sorokiniana* mutants, cells were prepared for UV mutagenesis as described for the kill curve. Cells were exposed to UV light for 7 min resulting in 97%-98% kill. UV-exposed cells were diluted to 2-3 cells/300 µL of SUN-glucose medium and 300 µL were aliquotted into each well of a 96-well microtiter plate (Sarstadt, uncoated, flat bottom). After 4 d incubation in the dark followed by 2 d incubation under fluorescent light, the number of colonies in each well of the microtiter plate was counted and recorded. A direct copy of the 96-well plate was prepared by transferring 20 µL of cells from each well of the first plate to SUN-glucose medium in the wells of a second plate. Since cells in the first plate were killed by the screening procedure, the second 96-well plate was used for retrieval of possible cell wall defective mutants. The first plate was centrifuged (5 min, 1,000*g*, 4°C) in a Beckman DU 640 centrifuge (adapted for 96-well plates), and supernatants were discarded. Cells in each well were resuspended in 300 µL of CHP in buffer. The first plate was incubated in the dark while shaking at 900 rpm at 35°C for 16 h. The first plate was centrifuged for 5 min, supernatants removed, and pellets resuspended in 200 µL of 1% NP-40 (Sigma). The first plate was centrifuged for 10 min and wells were observed for green supernatants (putative cell wall defective mutants).

Putative mutant cells, maintained in the second plate, were diluted and distributed so there was approximately 1 cell/well in a third microtiter plate. The cells from the third plate were grown and screened as they were for the first plate. A fourth plate was maintained as a copy of the third plate. Putative mutants retrieved from the fourth plate were again diluted to 1 cell/well into a fifth plate and grown and screened as described for the first and third plates. Each final mutant culture was derived from a colony which was, identified as a mutant on the fifth plate and retrieved from the sixth, or copy, plate. Using this procedure, cells from plates 1, 3, and 5 were screened for the mutant phenotype. Putative mutants were recovered from plates 2, 4, and 6. Mutant cultures were maintained on SUN and SUN-glucose slants under fluorescent light.

Using methods described in previous sections, putative mutants were analyzed for their growth characteristics, microscopic appearance, susceptibility of whole cells to treatment with polysaccharide-degrading enzymes, and cell wall composition.

RESULTS

C. sorokiniana Cell Wall Characterization

Polysaccharide Analysis

Qualitative data on *C. sorokiniana* cell wall polysaccharide composition have been reported previously. The strain of *C. sorokiniana* used in this laboratory has been maintained for about 35 years. The culture has been periodically streaked for isolation and colonies have been selected for future cultures based upon a fast growth rate. To ensure cell wall composition was still similar to that reported for other strains of *C. sorokiniana*, a complete analysis of the *C. sorokiniana* IIIB2NA #7 wall was conducted.

Purified *C. sorokiniana* cell walls were acid hydrolyzed to allow for release and subsequent identification of cell wall monosaccharides. Mild acid treatment in TFA hydrolyzes matrix polysaccharides which can be destroyed under stronger acid conditions. Stronger acids, HCl and H_2SO_4, are used to hydrolyze glucosamine polymers (acetylated and unacetlyated) and cellulose, respectively. Because neither glucose nor reducing sugars were detected in the H_2SO_4 hydrolysate, they were not analyzed using GC-MS.

Approximately 53% of the dry weight of the *C. sorokiniana* cell wall was released as monosaccharides (as analyzed by GC-MS) after TFA hydrolysis. The primary monosaccharide released, rhamnose, contributed to 72% of the TFA hydrolysate or 38% of the cell wall dry weight. Galactose made up 13% of the TFA hydrolysate or 6.7% of the cell wall dry weight. Monosaccharides that each contributed to at least 3% of the hydrolysate were glucuronic acid, mannose, and xylitol. Trace amounts of xylose, glucose, and glucosamine were also identified (Figure 1, Table 1). Hydrolysis of TFA-extracted walls with HCl released an additional 35% of wall dry weight. Glucuronic acid, a major component of pectin, made up 40% of the HCl hydrolysate or 14% of cell wall dry weight. Galactose, rhamnose, and xylitol contributed significantly to the HCl hydrolysate making up 22%, 18%, and 9.3% of the hydrolysate, respectively. The remaining monosaccharides were identified as mannose, glucose, glucosamine, and galactosamine. Each contributed to less than 4% of the monosaccharides released by HCl hydrolysis. An additional peak was present on the gas chromatogram of the HCL hydrolysate, and was identified as an amino sugar using mass spectrometry. The concentration of the unidentified amino sugar could not be determined without comparison of a known external standard to inositol.

An analysis of combined data for TFA and HCl hydrolyses showed rhamnose as the major monosaccharide contributing to

Figure 1. Identification of the monosaccharides released through acid hydrolysis of the *C. sorokiniana* cell wall. Purified cell walls were hydrolyzed in 2 N TFA for 6 h at 100°C followed by hydrolysis in 6 N HCl for 18 h at 110°C. Percentage of hydrolysate represented by each monosaccharide in TFA hydrolysate, HCl hydrolysate, and the two acid hydrolysates combined, is presented.

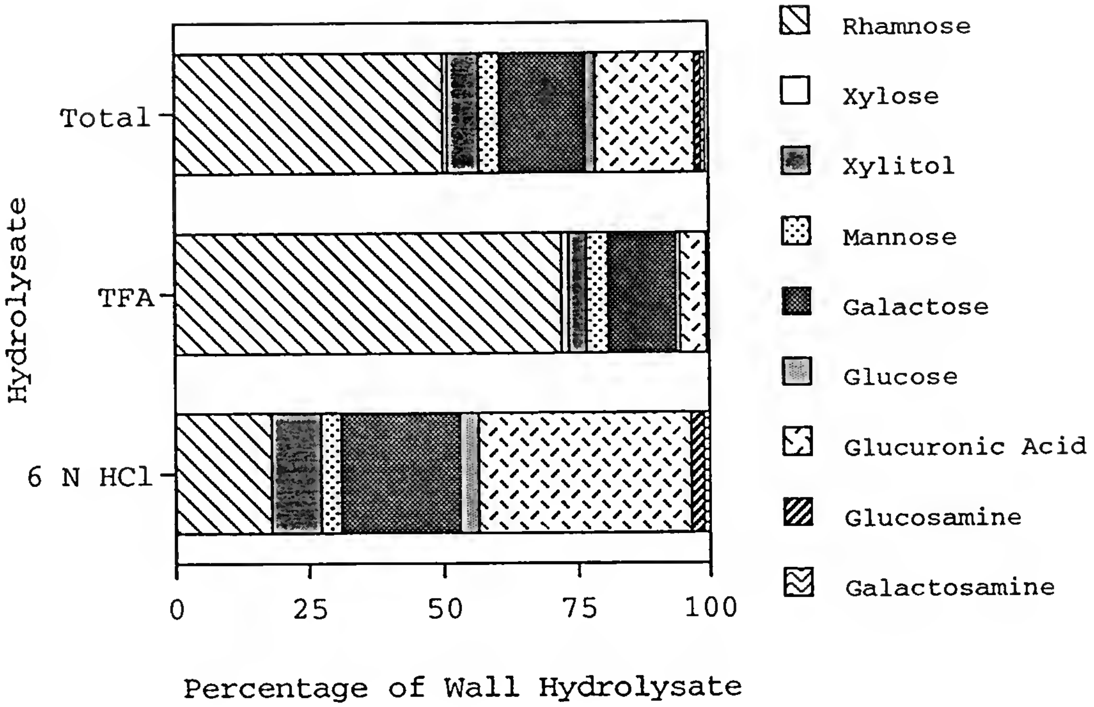

Total
TFA
6 N HCl
Hydrolysate
0
25
50
75
100
Percentage of Wall Hydrolysate
Rhamnose
Xylose
Xylitol
Mannose
Galactose
Glucose
Glucuronic Acid
Glucosamine
Galactosamine

Table 1. GC-MS determination of constituent monosaccharide composition of the *C. sorokiniana* cell wall

Monosaccharide	TFA Hydrolysate	HCl Hydrolysate	Total Hydrolysate
	Percent of Cell Wall Dry Weight		
Rhamnose	38	6.2	44
Xylose	0.75	0	0.70
Xylitol	1.8	3.3	5.1
Mannose	2.1	1.3	3.4
Galactose	6.7	7.6	14
Glucose	0.45	1.2	1.7
Glucuronic Acid	2.6	14	17
Glucosamine	0.10	0.92	1.0
Galactosamine	0	0.68	0.68
Total Polysaccharide[a]	50	33	83

[a] Total polysaccharide was estimated by calculating mol of monosaccharide and subtracting weight of one mol of water lost upon formation of a glycosidic bond for every two mol of monosaccharide.

44% of the cell wall dry weight. Other major monosaccharides were glucuronic acid and galactose making up 17% and 14% of wall dry weight, respectively. Xylitol and mannose made up at least 3% of the wall and xylose, glucosamine, and galactosamine each contributed to 1% or less of the wall dry weight (Table 1). Results of analyses of acid hydrolysates using spectrophotometric assays are presented in Table 2. A low amount of uronic acid and amino sugar was identified in acid hydrolysates of *C. sorokiniana*. Glucose was identified in the H_2SO_4 hydrolysate of *C. fusca* but not that of *C. sorokiniana*.

As determined by the phenol sulfuric acid assay, 25% of the *C. sorokiniana* cell wall dry weight was extracted as carbohydrate with 1 N NaOH. Glycosidic bonds are not as labile in alkali as they are in acid. As a result, oligosaccharides as opposed to monosaccharides are often released by alkaline hydrolysis. Proton decoupled [^{13}C] NMR is useful in determination of characteristics of polysaccharides.

A [^{13}C] NMR spectrum of an ethanol precipitate of an alkaline extract of the *C. sorokiniana* wall had about 54 signals (Figure 2). A similar spectrum was obtained even after dialysis of the sample in 1,000 mwco dialysis tubing against dH_2O. All signals were relative to a TSP standard.

Anomeric carbons resonate from approximately 90-110 ppm. Carbons with secondary hydroxyl groups compose the most

Table 2. Colorimetric analyses of acid hydrolysates of *Chlorella* cell walls

Species	Acid Hydrolysate	Percentage of Cell Wall Dry Weight: Total Carbohydrate[a]	Glucose[b]	Amino Sugar[c]	Uronic Acid[d]
C. sorokiniana					
	TFA	ND[e]	ND	ND	1.9 ± 0.06
	H_2SO_4	0.07 ± 0.01	0.05 ± 0.07	0 ± 0	ND
	HCl	0.30 ± 0	0.08 ± 0	1.6 ± 0.15	ND
C. fusca					
	TFA	ND	ND	ND	0.70 ± 0.01
	H_2SO_4	8.4 ± 0.73	8.9 ± 2.6	0 ± 0	ND
	HCl	0.02 ± 0.01	0 ± 0	0 ± 0	ND

[a] Total carbohydrate was measured using the phenol sulfuric acid assay

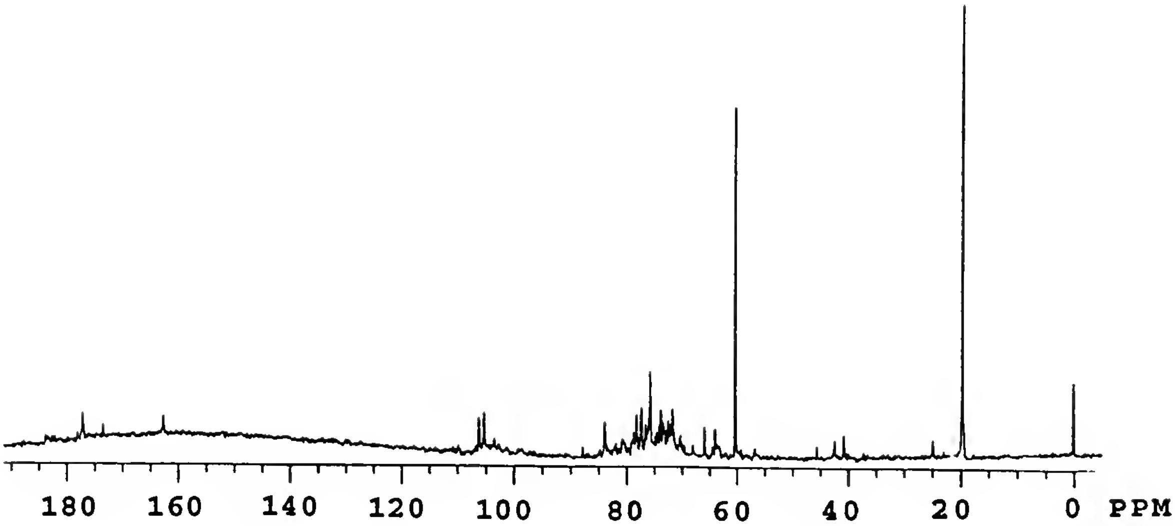
[b] Glucose was measured using the glucose oxidase assay

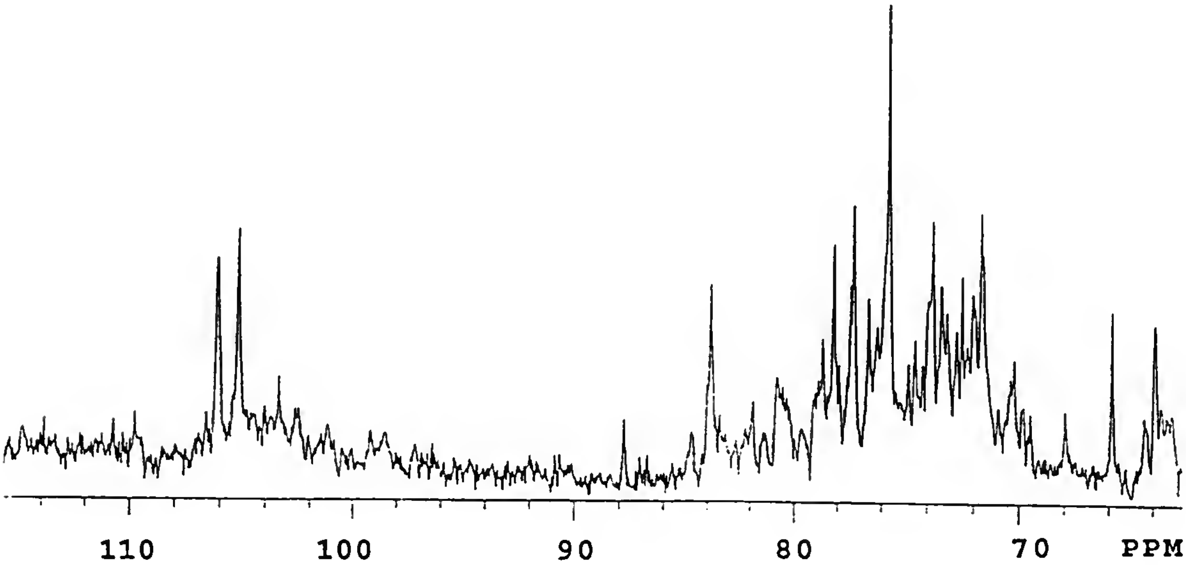
[c] Amino sugars were measured using the Elson Morgan assay

[d] Uronic acids were measured using the metahydroxy diphenyl assay

[e] ND not determined

Figure 2. Proton-decoupled [^{13}C] NMR profile of the ethanol precipitate of an alkaline-soluble fraction of the *C. sorokiniana* cell wall.


180
160
140
120
100
80
60
40
20
0
PPM


110
100
90
80
70
PPM

complex portion of the [^{13}C] NMR spectrum of polysaccharides from approximately 60-90 ppm. Two major signals at 105.3 and 106.0 ppm represent anomeric carbons of two saccharides. A signal at 103.3 ppm represents a lower concentration of a third saccharide. It is likely that secondary hydroxyl groups of xylitol are represented by the major signal at 75.5 ppm.

Characteristics other than saccharide identification can be determined using [^{13}C] NMR. Hydroxy methyl groups on C_6 resonate at 65-69 ppm. Two signals at approximately 64.9 and 66.0 ppm represent hydroxy methyl groups. It is not known with which saccharides they are associated. Carbons in glycosidic bonds can have characteristic chemical shifts. Signals at 68.0 ppm and 70.2 ppm may represent C_6 residues coupled through a glycosidic 1,6-linkage. The signal at 88 ppm also represents a carbon involved in a glycosidic linkage.

In Figure 2, signals at 60 ppm and 20 ppm represent carbons from ethanol that was not completely removed during sample preparation. In other samples where ethanol was copletely removed (data not presented), only a small signal at 20 ppm persisted. Relatively minor signals at 20 ppm and 175-180 ppm represent methyl and carbonyl groups, respectively. Methyl groups may be the C_6 of rhamnose. Carbonyl groups may be C_6 of uronic acids. Conversely, these groups may be associated with proteins that were extracted with the polysaccharides.

Like TFA hydrolysis, alkaline hydrolysis solubilizes matrix polysaccharides and leaves the rigid portion of the wall unaffected. Because of harsh conditions under which glucosamine polymers are acid hydrolyzed, some destruction of glucosamine can occur during hydrolysis. Destruction of glucosamine results in an underestimation of glucosamine concentration. Alkaline-insoluble wall residue was analyzed by IR and elemental analysis to see how closely this cell wall fraction resembles an authentic glucosamine-containing polymer, chitosan.

An IR spectrum of a chitosan positive control was identical to a published spectrum of chitosan (Muzzarelli, 1973). Despite the chitosan spectrum having more dominant peaks than the cell wall sample at 1597, 1422, and 1154 cm^{-1}, the spectra are very similar and represent similar compounds (Figure 3). Because there are many types of chemical bonds that absorb in these regions, it is not possible to identify types of bonds causing the differences in the IR spectra. Elemental analysis (C, H, and N) of the alkaline-insoluble portion of the wall differs slightly from that of a chitosan positive control (Table 3). Chitosan has a C:N ratio of 1:6 as expected. The C:N ratio of the cell wall sample is 1:7. The three elements combined account for about 60% and 56% of chitosan standard and alkaline-extracted wall, respectively. If 4 mol of oxygen for each mole of nitrogen (as occurs for a polymer of authentic chitosan) are calculated into the final

Figure 3. IR spectra of chitosan (a) and residue remaining after alkaline extraction of the *C. sorokiniana* cell wall (b).

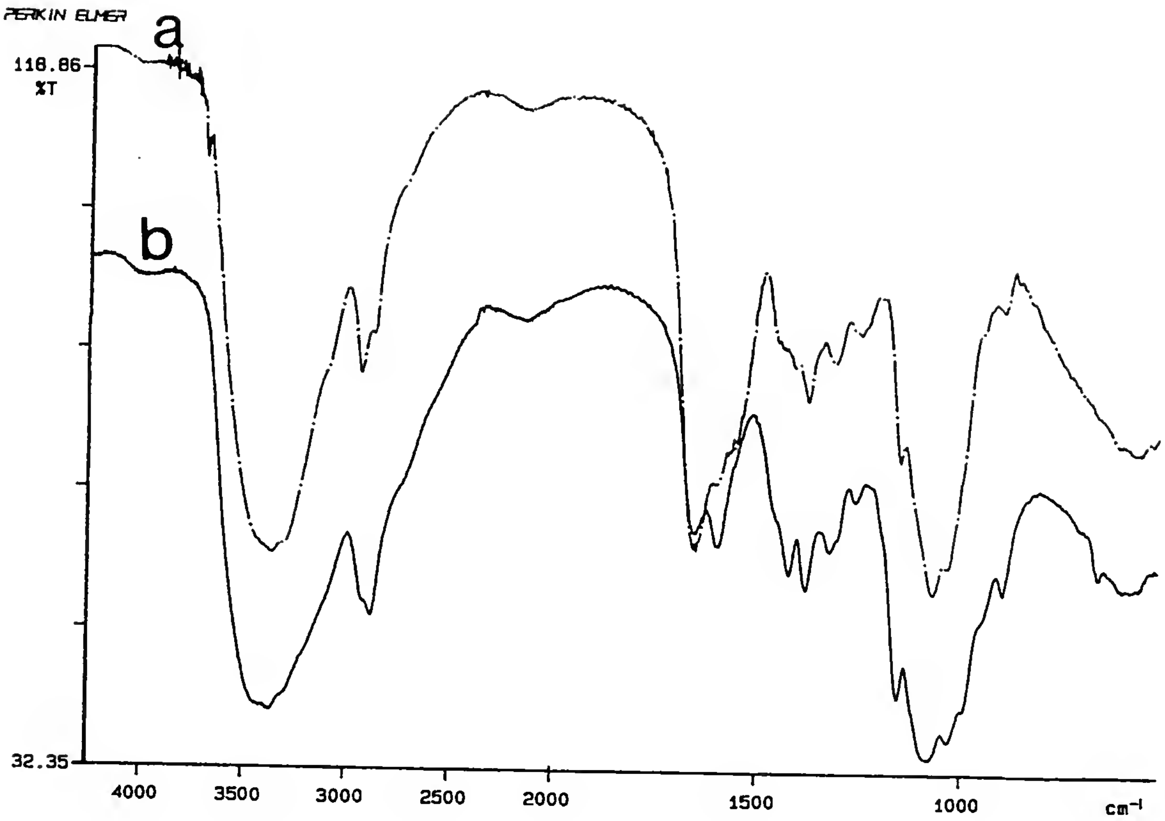

PERKIN ELMER
a
b
118.86
%T
32.35
4000
3500
3000
2500
2000
1500
1000
cm⁻¹

Table 3. Elemental analysis of chitosan and *C. sorokiniana* cell wall residues

Sample	Percentage of Sample Dry Weight			
	C	H	N	Total
Chitosan (expected)	40 (6)[a]	6.7 (12)	7.9 (1)	55
Chitosan (experimental)	44 (5.8)	7.4 (13)	8.2 (1.0)	60
C. sorokiniana Alkaline-extracted wall	42 (6.9)	6.4 (13)	7.2 (1.0)	56
C. sorokiniana Alkaline-extracted wall followed by acetolysis	52 (5.0)	6.9 (8.1)	12 (1.0)	71

[a] Numbers in parentheses represent mol of C, H, or N per mol of N in each sample.

wall weights, 97% of chitosan and 88% of alkaline-extracted wall weights are accounted for as C, H, N, and O. When 5 mol of oxygen are calculated into weight of alkaline-extracted wall, 97% of the wall dry weight is accounted for as C, H, N, and O.

Protein and Amino Acid Analyses

Total cell wall protein was estimated from analyses of alkaline extracts of purified cell walls. The Bradford and Lowry protein assays were performed on the extracts. Protein concentrations of the alkaline extracts were compared with those estimated from automated amino acid analysis of an HCl hydrolysate of cell wall proteins. Lowry and automated analyses yielded similar cell wall protein concentrations of 17%± 1.4 and 17% of the cell wall dry weight, respectively. The Bradford assay, in which protein was calculated as 9.0% ± 0.72% of the cell wall dry weight, had a consistent 53% underestimation of protein concentration for *C. sorokiniana* cell wall proteins. Amino acid analysis of a 6 N HCl extract of the wall revealed that glycine and alanine concentrations were slightly higher than that of other amino acids (Table 4). Estimations of lysine concentration were not repeatable between cell wall samples from same or different batches of purified cell walls. It is likely there is a compound that interferes with the elution of lysine from the column during gas chromatography (Nancy Denslow, personal communication). Therefore, calculation of lysine content was

Table 4. Amino acid composition of *C. sorokiniana* cell wall proteins

Amino acid	mol%
Hyp	0.020
Asx	8.33
Thr	6.59
Ser	3.74
Glx	5.63
Pro	6.60
Gly	7.90
Ala	8.18
Val	6.99
Met	0.69
Ile	3.60
Leu	5.26
Tyr	6.45
Phe	6.30
His	3.67
Arg	5.93

omitted from final amino acid data presented. Hydroxyproline was present in a very low concentration and cysteine and tryptophan concentrations were not estimated.

Analyses for Other Cell Wall Compounds

Sporopollenin

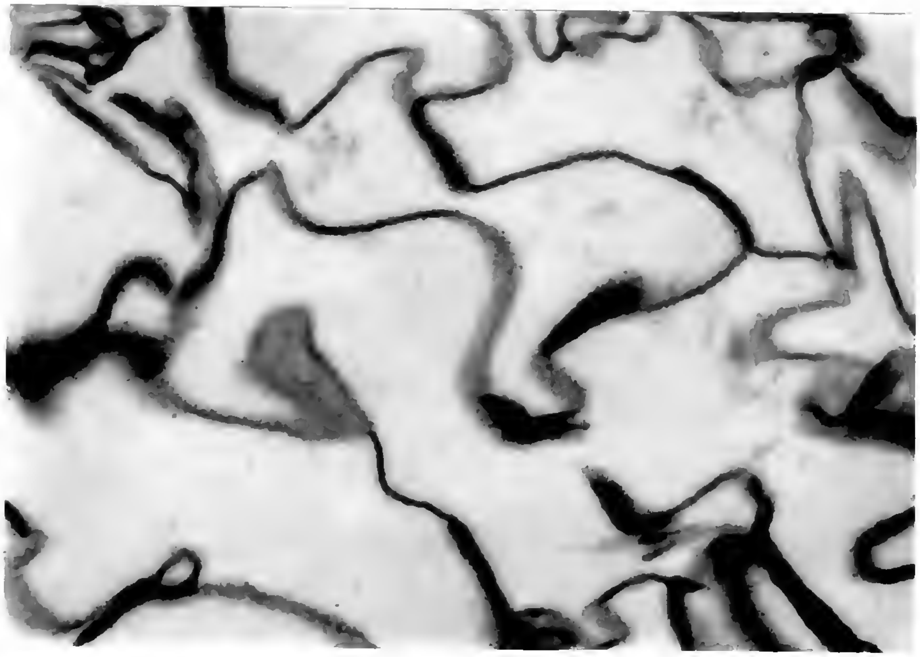
Because of the presence of an acetolysis-resistant fraction in both whole *C. sorokiniana* cells and purified cell walls, the cell wall was suspected to contain sporopollenin. *C. fusca* walls, which contain sporopollenin, also had an acetolysis-resistant residue whereas walls of two species without sporopollenin, *C. ellipsoidea* and *C. saccharophila* dissolved even before the acid solution was heated (Table 5). Using TEM, it was confirmed that the portion of the *C. sorokiniana* cell resistant to acetolysis was the wall, or a portion thereof. Only the trilaminar sporopollenin-containing layer remained after acetolysis.The *C. sorokiniana* wall did not exhibit a decrease in cell wall thickness following acetolysis treatment (Figure 4).

C. sorokiniana cells do not possess an outer trilaminar wall layer as is seen in algae that have sporopollenin (e.g., *C. fusca*). The 80 µm *C. sorokiniana* wall was somewhat thinner than the 114 µm *C. fusca* wall. Instead of having an outer trilaminar layer, the *C. sorokiniana* wall is sometimes

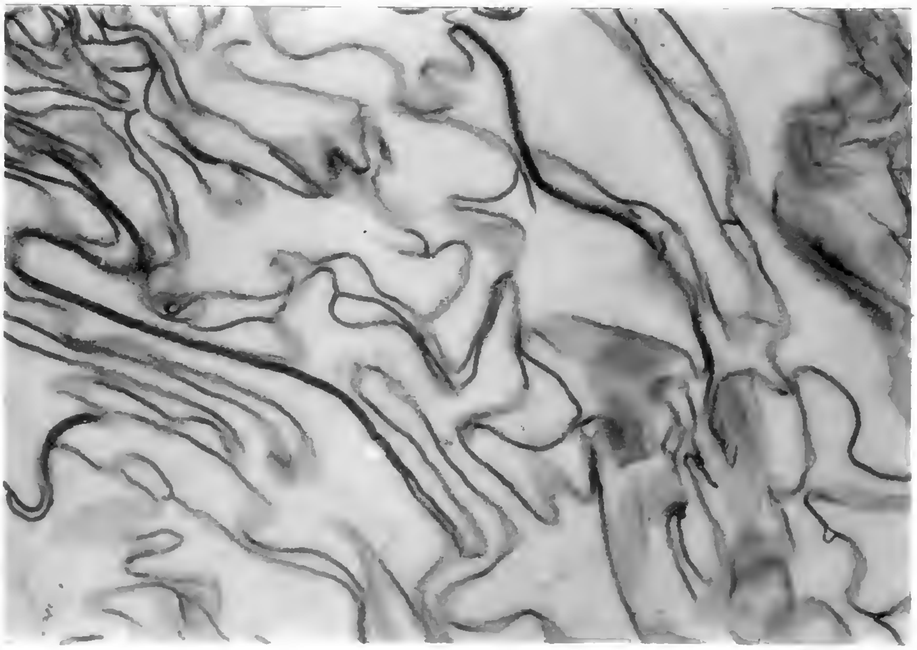
Table 5. Acetolysis and phosphorolysis of purified *Chlorella* cell walls

Cells	Conditions	% Cell Wall Dry Weight Remaining
C. ellipsoidea	acetolysis	0
C. saccharophila	acetolysis	0
C. fusca	acetolysis	34.8 ± 3.6
	TFA hydrolysis followed by acetolysis	14.4 ± 14.6
	alkaline extraction followed by phosphorolysis	5.25 ± 0.66
C. sorokiniana	acetolysis	21.5 ± 3.0
	TFA hydrolysis followed by acetolysis	8.43 ± 5.4
	alkaline extraction followed by acetolysis	0.71 ± 0.69
	alkaline extraction followed by phosphorolysis	0

Figure 4. Acetolysis resistant residues of *C. sorokiniana* (a) and *C. fusca* (b) cell walls. Enlargement X50,000.



a



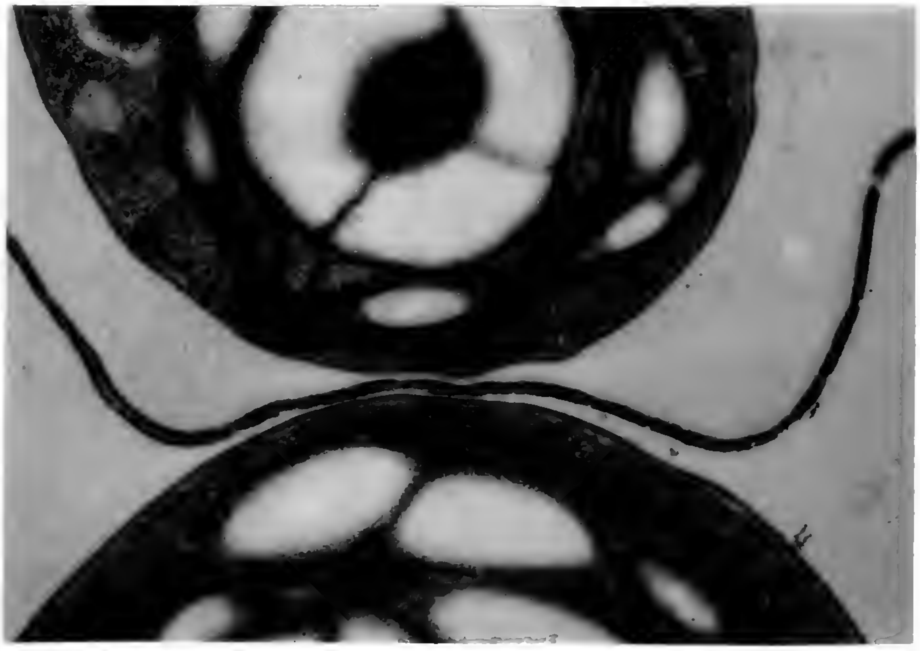
b

seen as having a single outer monolayer and a thicker inner layer. Despite convincing evidence of a two-layered wall, a single homogeneous wall layer was observed more frequently, in electron micrographs (Figure 5).

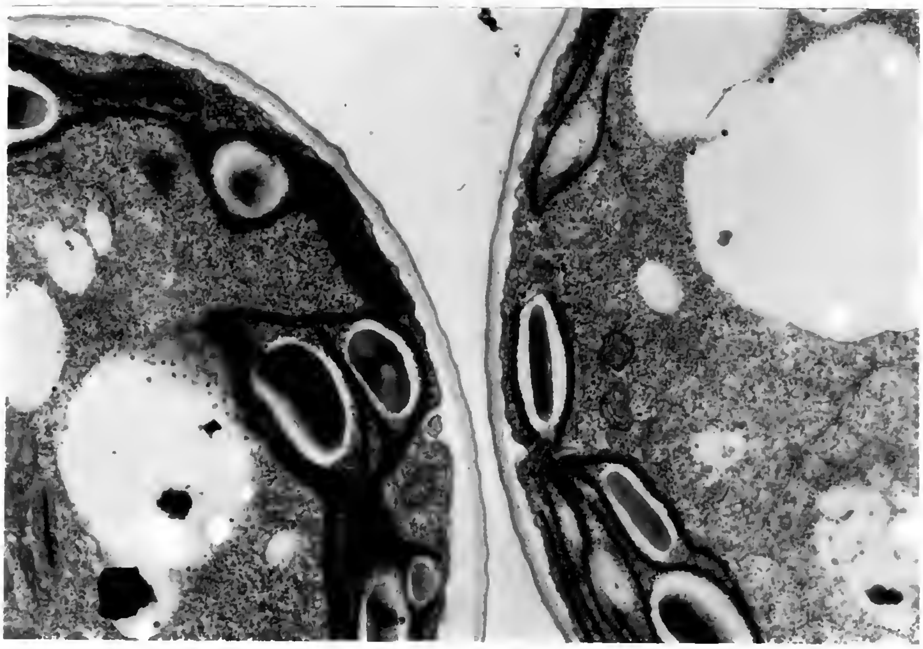
Cell wall autofluorescence is another characteristic that indicated the presence of sporopollenin in the *C. sorokiniana* wall. Yellow autofluorescence upon excitation with blue light is present in both the *C. sorokiniana* and the *C. fusca* walls and not present in the sporopollenin-lacking *C. ellipsoidea* wall (Figure 6). The acetolysis-resistant residues of *C. sorokiniana* and *C. fusca* are autofluorescent.

There is some indication that autofluorescence of the *C. sorokiniana* wall may be due to a compound other than sporopollenin. The alkaline resistant cell wall residue had faint autofluorescence; whereas an ethanol precipitate of the alkaline solubilized portion of the wall had stronger autofluorescence. Autofluorescence was also observed for commercially prepared chitin and chitosan. A definitive test for sporopollenin is its insolubility when incubated in alkali followed by incubation in concentrated phosphoric acid (i.e., phosphorolysis). Unlike *C. fusca* cell walls, *C. sorokiniana* cell walls are completely solubilized by phosphorolysis and, therefore, do not contain sporopollenin (Table 5).

Figure 5. Transmission electron micrographs of *C. sorokiniana* and *C. fusca* cell walls. The *C. sorokiniana* cell wall often has a single homogeneous cell wall layer as shown in the daughter wall (a). In some cell preparations, the wall appears to be constructed of two distinct layers as shown in the mother wall (a). The *C. fusca* wall has of a thick inner layer and thinner outer-trilaminar sporopollenin-containing layer (b). Enlargement X40,000.

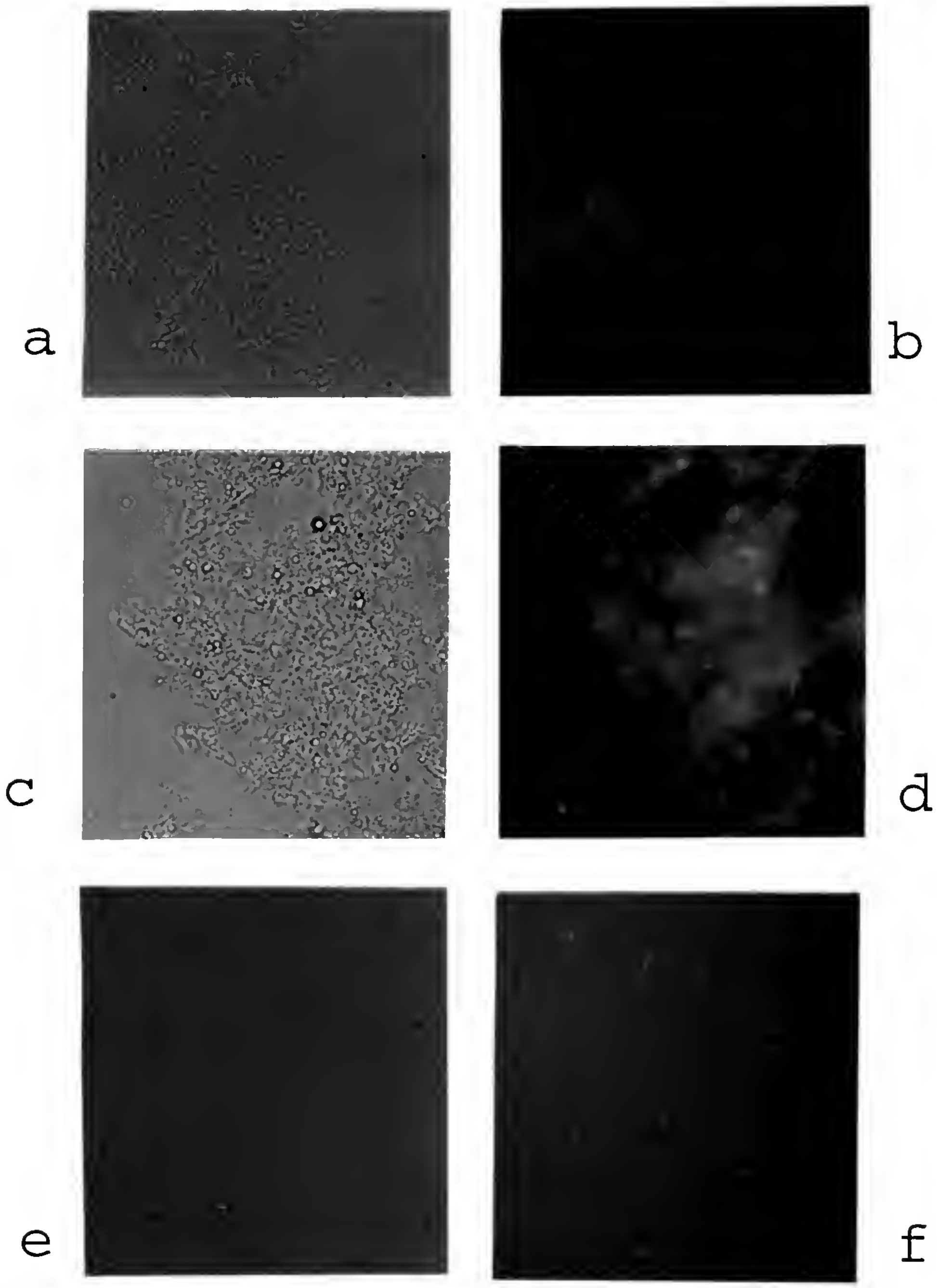


a



b

Figure 6. Autofluorescence of purified *Chlorella* cell walls. Cell wall samples are *C. sorokiniana* under light microscopy (a), *C. sorokiniana* under fluorescence microscopy (b), *C. fusca* under light microscopy (c), *C. fusca* under fluorescence microscopy (d), *C. ellipsoidea* under light microscopy (e), and *C. ellipsoidea* under fluorescence microscopy (f). Enlargement X1200.


a
b
c
d
e
f

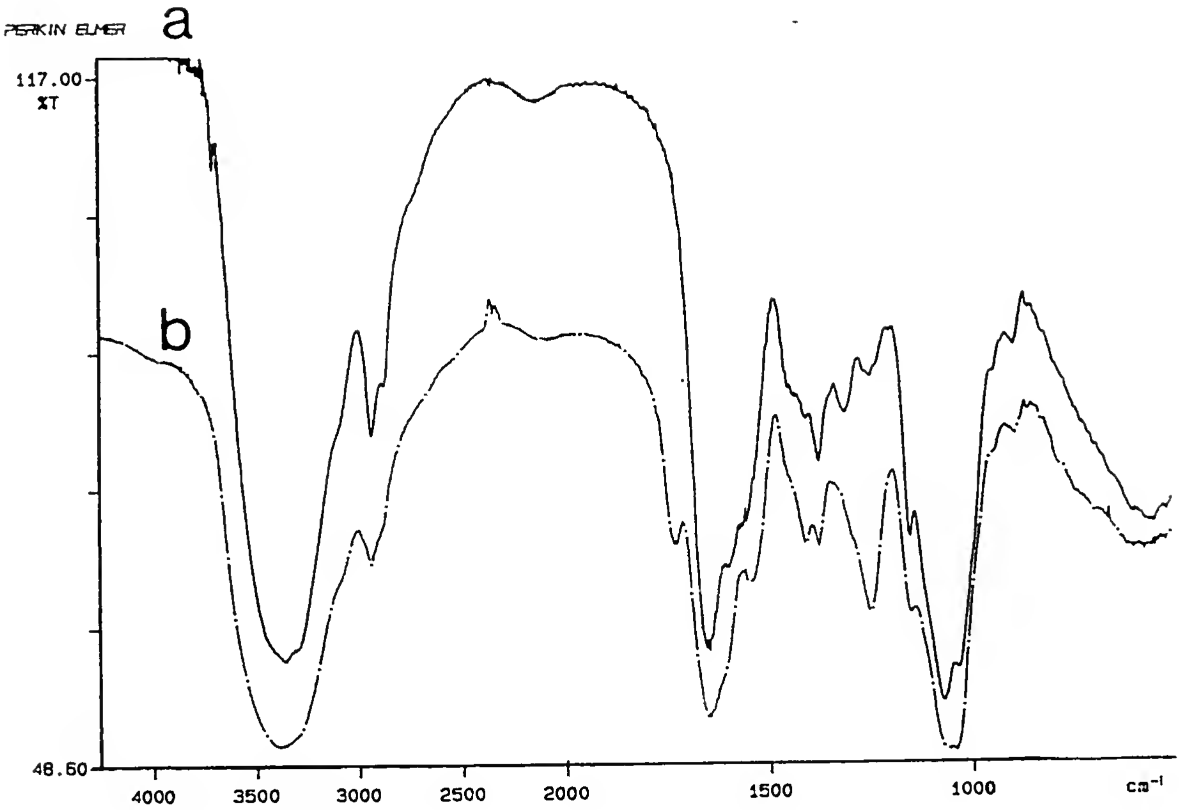
Phenolic compounds

Purified cell walls from *C. ellipsoidea, C. saccharophila, C. fusca,* and *C. sorokiniana* were subjected to a qualitative phloroglucinol test for lignin. Pine sawdust was used as a positive control. Pine sawdust mixed with phloroglucinol reagent immediately produced a dark magenta color, a positive test for lignin. *Chlorella* walls all gave colorless, negative results.

Treatment of *C. sorokiniana* cell walls with 1 N NaOH was rigorous enough to extract most phenolic compounds. A UV absorption spectrum and NMR analysis were performed on a butanol extract of the alkaline extract. The absorption spectrum had two absorption maxima, at 280 nm and 210 nm indicating the presence of protein. Absorption peaks, representing other aromatic compounds, were not present. The NMR spectrum had no major peaks that indicated the presence of phenolic or related substances in a concentration greater than approximately 2 µmol mL^{-1}. Protein (identified by the absorption spectrum) was present at a quantity insufficient for measurement using $[^{13}C]$ NMR.

An infrared spectrum of purified cell walls was examined for the presence of phenolic compounds (Figure 7). Skeletal bonds of aromatic compounds produce strong absorption bands from 1600 cm^{-1} to 1300 cm^{-1}. Since this region of the spectrum was complex, it was not useful in identification of

Figure 7. IR spectra of purified *C. sorokiniana* cell walls. Alkaline resistant cell wall residue (a) and total cell wall (b).


PERKIN ELMER
a
b
117.00
%T
48.50
4000
3500
3000
2500
2000
1500
1000
cm⁻¹

aromatic compounds. Aromatics also have strong absorption from 900 cm^{-1} to 650 cm^{-1}. Since the *C. sorokiniana* wall was void of absorption peaks in this region, an IR spectrum did not provide evidence for phenolic compounds in the cell wall.

Acetolysis-resistant portion of the *C. sorokiniana* cell wall

Polysaccharides of the *C. sorokiniana* wall contain a relatively high concentration of rhamnose. Glycosidic bonds involving rhamnose molecules are sensitive to TFA hydrolysis, but are fairly resistant to acetolysis. To determine if a TFA-hydrolyzable cell wall fraction contributes to cell wall resistance to acetolysis, TFA hydrolysis of the cell wall was performed prior to acetolysis. TFA hydrolysis of *C. sorokiniana* cell walls prior to acetolysis decreased the amount of acetolysis resistant wall fraction from 22% for acetolysis alone to 8.4% for TFA hydrolysis plus acetolysis. Alkaline extraction of the wall, which removed many polysaccharides, proteins, and possibly other compounds prior to acetolysis, decreased the acetolysis-resistant residue to less than 1% of the cell wall dry weight (Table 5).

To determine whether chitin and chitosan are solubilized by acetolysis, acetolysis of these compounds was performed. Resistance of these polymers to acetolysis would leave open a possibility that the glucosamine-containing polymer in the *C. sorokiniana* wall may contribute to cell wall acetolysis resistance. Chitin was solubilized during boiling in the acetolysis solution. Although chitosan survived boiling in the acetolysis solution, it was solubilized during a subsequent sodium acetate wash step.

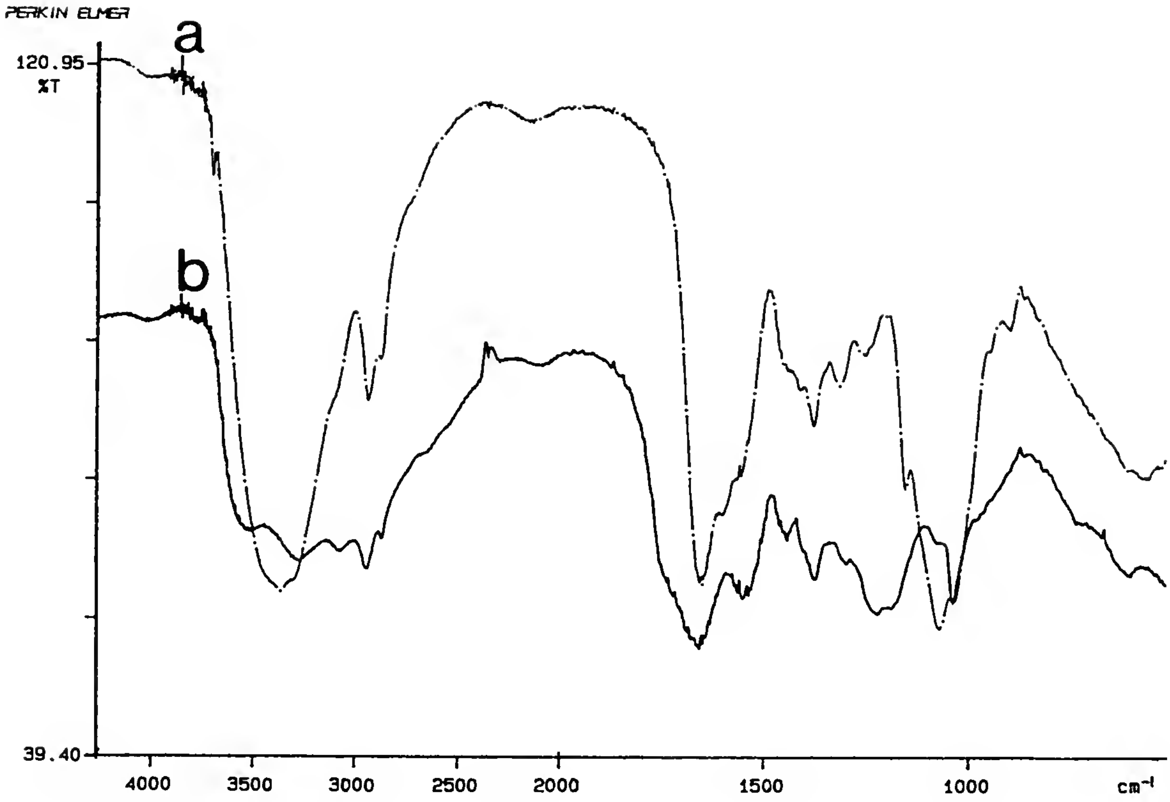
In an attempt to gain additional evidence as to identity the acetolysis resistant residue, an infrared spectrum and elemental analysis were performed on the residue. An IR spectrum of an acetolyzed cell wall resembled that of the alkaline resistant wall, with some differences in peak location and intensity (Figure 8). An increase in absorbance around 1720 cm^{-1} indicated the presence of carbonyl groups formed by acetolysis. Other changes could not be characterized due to complexity of the spectrum. Elemental analysis of acetolysis resistant residue showed that it has a higher C:N ratio than either chitosan or alkaline resistant wall residue (Table 3). If one or two molecules of oxygen are calculated per mol of nitrogen, 83% and 98% of the wall, respectively, is accounted for.

Effect of Hydrolytic Enzymes on the *C. sorokiniana* Cell Wall

Effect of Enzymes on Whole Cells

Protoplasts were not produced by any polysaccharide-degrading enzymes either alone or in combinations. When cells were treated with CHP, osmotic lability of cells was not apparent for at least 10 h. After 16 h of incubation, when cells were diluted with water on a microscope slide,

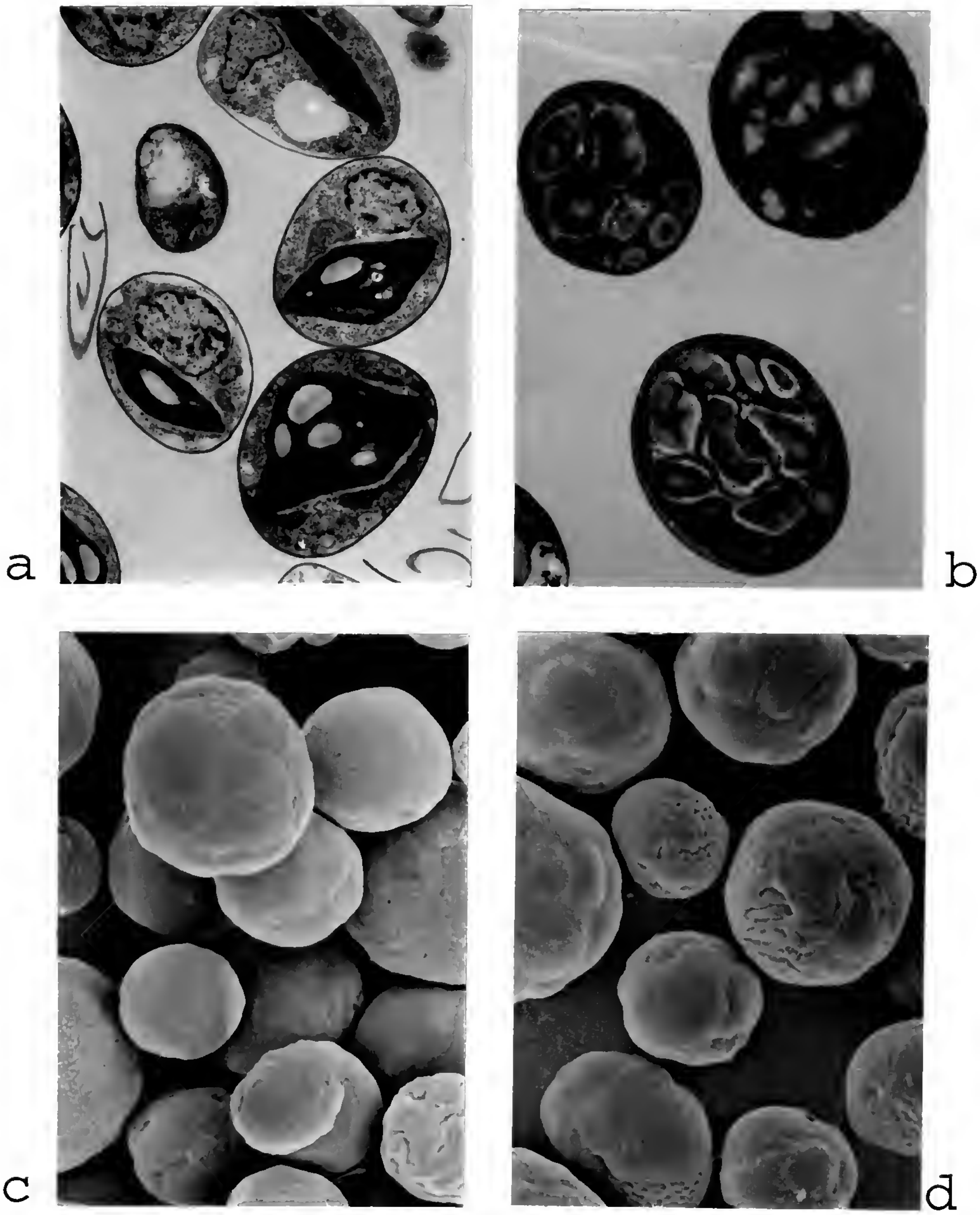
Figure 8. IR spectra of the *C. sorokiniana* alkaline extracted cell wall residue (a) and alkaline extracted cell wall residue after subsequent acetolysis treatment (b).


PERKIN ELMER
a
b
120.95
%T
39.40
4000
3500
3000
2500
2000
1500
1000
cm⁻¹

about 5-10% of cells ruptured and cell contents leaked partially or entirely out of one spot in the cell wall. The cell wall, void of its contents, was still visible by light microscopy. These cells had no apparent change in cell wall morphology. However, there appeared to be an increase in internal disorganization of the cells such as cytoplasmic and thylakoid membrane disruption. Therefore, it is likely that these cells were displaying signs of cell death rather than enzymatic cell wall degradation (Figure 9).

From attempts to make protoplasts by treatment of cells with a mixture of polysaccharide-degrading enzymes, cell homogenate, and a protease, inconsistent results were obtained. Partially synchronized cells (harvested 3 h and 45 min into the cell cycle) were treated with chitosanase, cell wall homogenate, and pronase. After 4 h of enzyme treatment in pH 6 buffer, a low percentage of cells (<5%) lysed in water. Unlike cells treated with carbohydrate-degrading enzymes that leaked out of a single spot in an otherwise intact cell wall, a cell wall was not apparent (by light microscopy) as these cells lysed in water. When cells were harvested 3 h into the cell cycle and treated with cell homogenate, CHP, chitosanase, and protease, approximately 30% of cells lysed as they were diluted with dH_2O on a slide. When treated with only homogenate and chitosanase, approximately 20% of cells lysed. Cell lysis was observed when enzyme solutions were prepared in pH 6, but not pH 7

Figure 9. TEM and SEM of *C. sorokiniana* cells untreated and treated for 16 h with CHP. TEM of untreated cells (a), TEM of enzyme-treated cells (b), SEM of untreated cells (c), and SEM of enzyme-treated cells (d). Enlargement X12,500.


a
b
c
d

sodium phosphate buffers. Cell lysis was still obtained when either homogenate or protease was omitted from the enzyme solution. These results represent data from three experiments performed on the same starting culture of daughter cells. However, in six subsequent experiments (one in which the same starting culture was used as in the first three experiments) success in cell lysis, and possible protoplast formation was not repeated.

Effect of Enzymes on Purified Walls

Although walls of intact *C. sorokiniana* cells were resistant to enzymatic degradation, purified cell walls were also tested for their sensitivity to enzymatic degradation for several reasons: (i) a quantitative assessment of cell wall degradation could be made on purified walls without a possibility of cell metabolites interfering with assay results, (ii) induced metabolic responses, such as wall protein cross-linking and production of compounds to decrease cell wall degrading enzyme activities could be ruled out as a sole reason for lack of cell wall enzymatic hydrolysis, and (iii) it could be determined whether or not an outer resistant cell wall layer protected an inner, enzyme-sensitive cell wall layer. Efforts could then be focused on determination of resistant outer wall layer composition. Commercially prepared cellulase, hemicellulase, and pectinase contained reducing sugars as fillers at a level sufficient to interfere with the phenol sulfuric acid assay. Soluble sugars were removed (90-100% decrease) by ultrafiltration. Reducing-sugar-free enzyme preparations retained activity as demonstrated by their ability to produce protoplasts from *C. ellipsoidea* cells.

C. ellipsoidea walls, treated with CHP, were used as a positive control. Combined enzymes released about 28% of the wall as carbohydrate (as measured by the phenol sulfuric acid assay), and each enzyme alone released slightly less. When *C. sorokiniana* walls were treated with CHP, approximately 6% of the wall was released as carbohydrate. This carbohydrate was equivalent to the amount of carbohydrate released from walls treated with pectinase alone. Cellulase and hemicellulase each released slightly less carbohydrate from purified walls (Table 6).

The phenol sulfuric acid assay does not measure amino sugars; the Elson-Morgan assay does measure amino sugars, but is too insensitive for this application. Therefore, the neocuproine assay for reducing sugars was used to measure the amount of reducing sugar released by treatment of cell walls with chitosanase.

Chitosanase, purified from *S. lividans* pRL207, degraded soluble chitosan more readily and more efficiently than it did insoluble chitosan (Figure 10). After a 2 h reaction, over 50% of soluble chitosan was released as reducing sugars. Chitosanase from *S. lividans* is an endolytic enzyme, degrading chitosan into mostly disaccharides and

Table 6. Percent degradation of purified cell walls subjected to enzymatic digestion

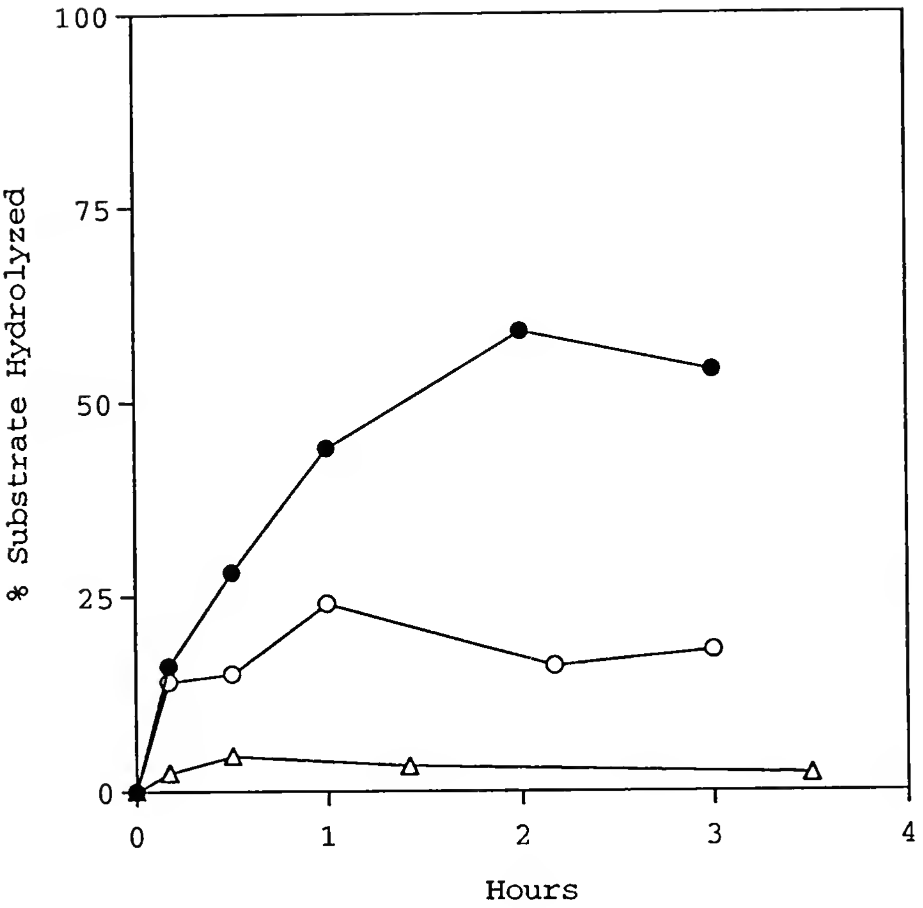
Substrate	Enzyme	Percent Dry Weight of Substrate Released[a]
C. ellipsoidea walls	CHP[b]	28 ± 1.8
	cellulase	21 ± 1.4
	hemicellulase	19 ± 1.4
	pectinase	18 ± 0
C. sorokiniana walls	CHP	6.0 ± 3.9
	cellulase	3.8 ± 0.35
	hemicellulase	2.1 ± 0.070
	pectinase	6.5 ± 0.28
	chitosanase	4.7 ± 0.65[c]
	CHP + chitosanase	4.4 ± 1.2[c]

[a] Phenol-sulfuric acid assay for total carbohydrate was used unless specified otherwise

[b] CHP stands for a mixture of cellulase, hemicellulase, and pectinase

[c] Neocuproine assay for reducing sugars

Figure 10. Kinetics of degradation of substrates by chitosanase from *S. lividans* pRL207. Substrates were provided as a 0.1% solution (v/v) or suspension (w/v). Substrates are identified as soluble chitosan (-●-), insoluble chitosan (-O–), and insoluble *C. sorokiniana* cell walls (-Δ-).


100
75
50
25
0
% Substrate Hydrolyzed
0
1
2
3
4
Hours

trisaccharides of glucosamine (Boucher, et al., 1992). Because this assay measures reducing sugars, and both monosaccharides and oligosaccharide are measured as one reducing sugar, it is likely maximum substrate degradation occurred. Approximately 20% of the insoluble chitosan was released in the same amount of time. When *C. sorokiniana* walls were treated with chitosanase, only 4% of the cell wall was degraded. For each of the substrates tested, no further increase in the amount of reducing sugar was observed beyond 3 h. The fluorescamine assay was employed to determine if primary amines, such as glucosamine, amino acids, and peptides, were released from compounds upon treatment of cell walls with single enzymes or combinations of enzymes. These data cannot be presented quantitatively, because fluorescamine derivatives of different compounds fluoresce at different intensities. Chitosan (solubilized in a HCl solution or insoluble) and BSA positive-control substrates did not react substantially with fluorescamine. Free glucosamine reacted with fluorescamine; a linear standard curve was constructed from a series of glucosamine standards.

Supernatants of chitosanase-treated purified *C. sorokiniana* cell walls exhibited little fluorescence in the presence of fluorescamine. Supernatants from walls treated with pronase, CHP, chitosanase and pronase, CHP and chitosanase, or CHP and pronase each exhibited a moderate intensity of fluorescence. A slightly higher intensity of fluorescence was observed from supernatants of walls treated with a mixture of CHP, chitosanase, and pronase or pronase followed by subsequent CHP or chitosanase treatment (Figure 11).

Effect of Enzymes on Thin Sections of Cells

Thin sections of *C. sorokiniana* and *C. fusca* cells were treated with enzyme mixtures to see if a cell wall layer was degraded. When *C. fusca* thin sections were treated with CHP, a decrease in electron density of the inner cell wall layer (cellulose layer) was apparent. With the same treatment of *C. sorokiniana* thin sections, no effect was observed (Figure 12). When *C. sorokiniana* thin sections were treated with a mixture of CHP, homogenate, chitosanase, and protease, there was no apparent decrease in electron density of the wall.

Attempts to Disrupt *C. sorokiniana* Cell Wall Biosynthesis

Attempts to Induce Ketocarotenoid Synthesis in *C. sorokiniana* and *C. fusca*

In *Chlorella*, the production of ketocarotenoids under nitrogen deficiency has been linked to the presence of acetolysis-resistant sporopollenin in the cell wall. Therefore, *C. sorokiniana* cells were tested for their ability to produce ketocarotenoids. After three days of incubation

Figure 11. Measurement of the enzymatic release of compounds containing primary amines from control substrates and the *C. sorokiniana* cell wall. Relative level of primary amines released into the supernatant was determined by the amount of fluorescence of the supernatant when reacted with fluorescamine. Control substrates with and without enzyme treatment, as specified (□), *C. sorokiniana* cell walls treated with single or simultaneously with multiple enzymes (▨), or *C. sorokiniana* walls with sequential enzyme treatment (■).

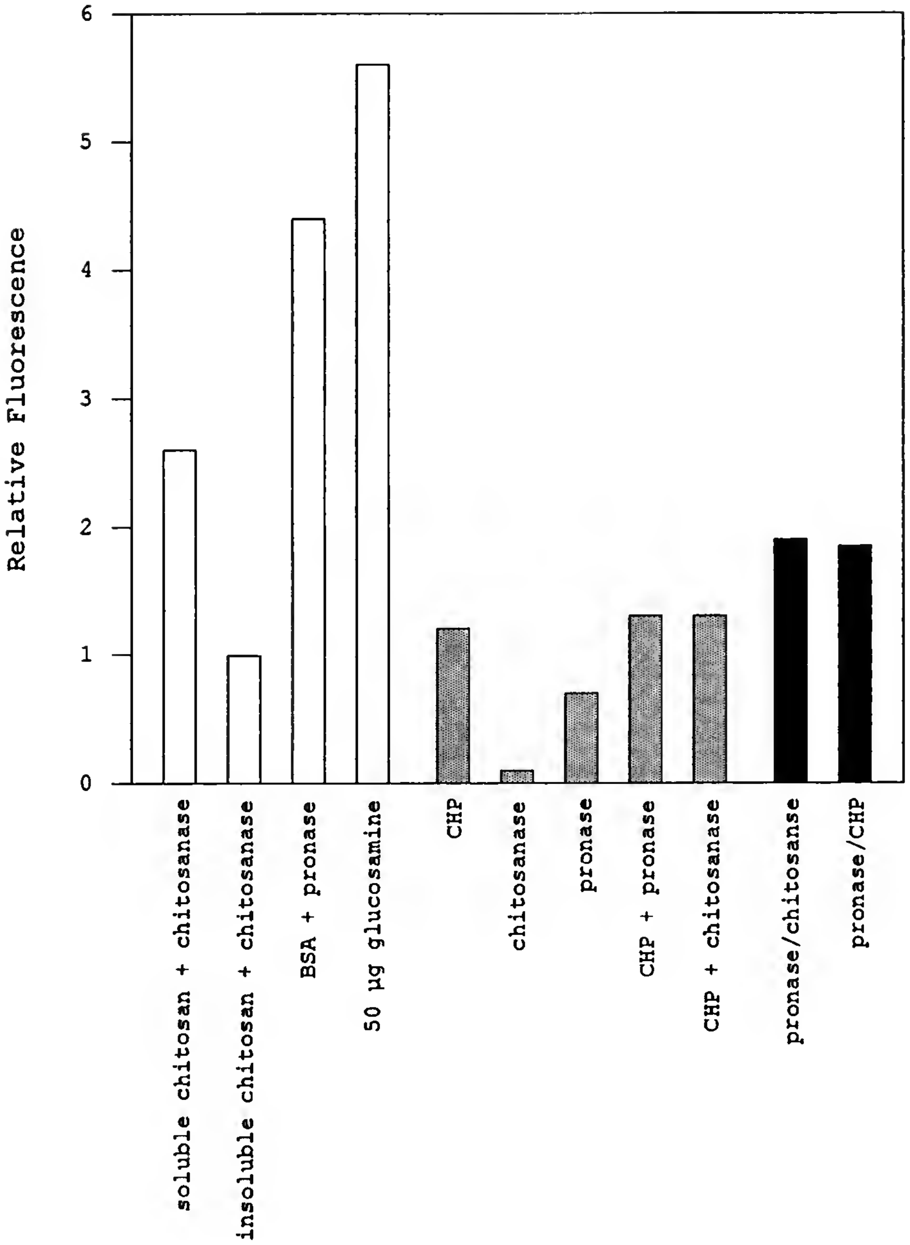
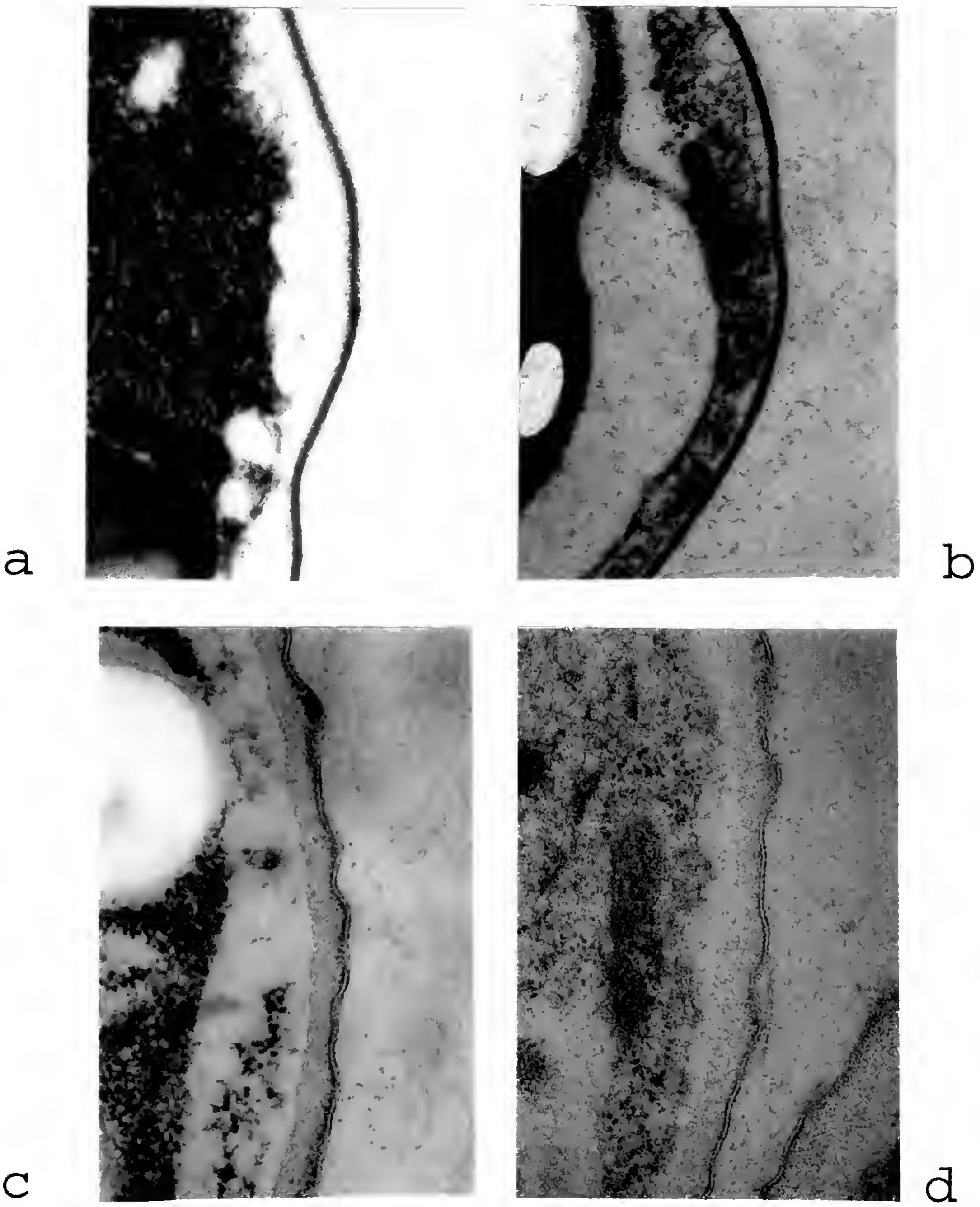

Relative Fluorescence
0
1
2
3
4
5
6
soluble chitosan + chitosanase
insoluble chitosan + chitosanase
BSA + pronase
50 µg glucosamine
CHP
chitosanase
pronase
CHP + pronase
CHP + chitosanase
pronase/chitosanse
pronase/CHP

Figure 12. TEM of thin sections of *Chlorella* cells treated with CHP after fixation and embedding of cells in Spurr's plastic. Untreated *C. sorokiniana* cells (a), *C. sorokiniana* cells treated with CHP (b), untreated *C. fusca* cells (c), and *C. fusca* cells treated with CHP (d). Enlargement X50,000.


a
b
c
d

of cultures in nitrogen-deficient SUN medium, the *C. sorokiniana* culture was colorless and did not produce ketocarotenoids. Microscopically, cells were small and possibly dead. Similar results were obtained whether *C. sorokiniana* cells were cultured at 38.5°C or 25°C. The *C. fusca* culture was yellow-orange and cells possessed an orange pigment that had the same R_f on TLC as did a canthaxanthin standard.

Effect of Norflurazon on *C. sorokiniana* Cell Walls

An inhibitor of carotenoid biosynthesis, norflurazon, was used to test whether or not the acetolysis resistant portion of the *C. sorokiniana* cell wall was derived from carotenoids. To protect *C. sorokiniana* cells from damaging effects of light at reduced carotenoid concentration, they were grown heterotrophically in the dark. Cultures supplemented with norflurazon had a decrease in growth rate relative to the amount of norflurazon added (Figure 13). Cultures supplemented with 1 µM norflurazon had a doubling time of 7.1 h as opposed to 6.3 h for an unsupplemented culture. Total carotenoid content of cells grown in 1 µM norflurazon was only 28% of that of cells grown without norflurazon (Table 7). The carotenoid profile of *C. sorokiniana* cell extracts was consistent with that of *C. zofingiensis*. Dominant pigments were lutein, violaxanthin,

Figure 13. Growth of *C. sorokiniana* cells in cultures containing norflurazon. *C. sorokiniana* cells were grown heterotrophically in the dark with the following concentrations of norflurazon; unsupplemented (-O-), 1 µM (-●-), 2 µM (-Δ-), and 3 µM (-▲-).

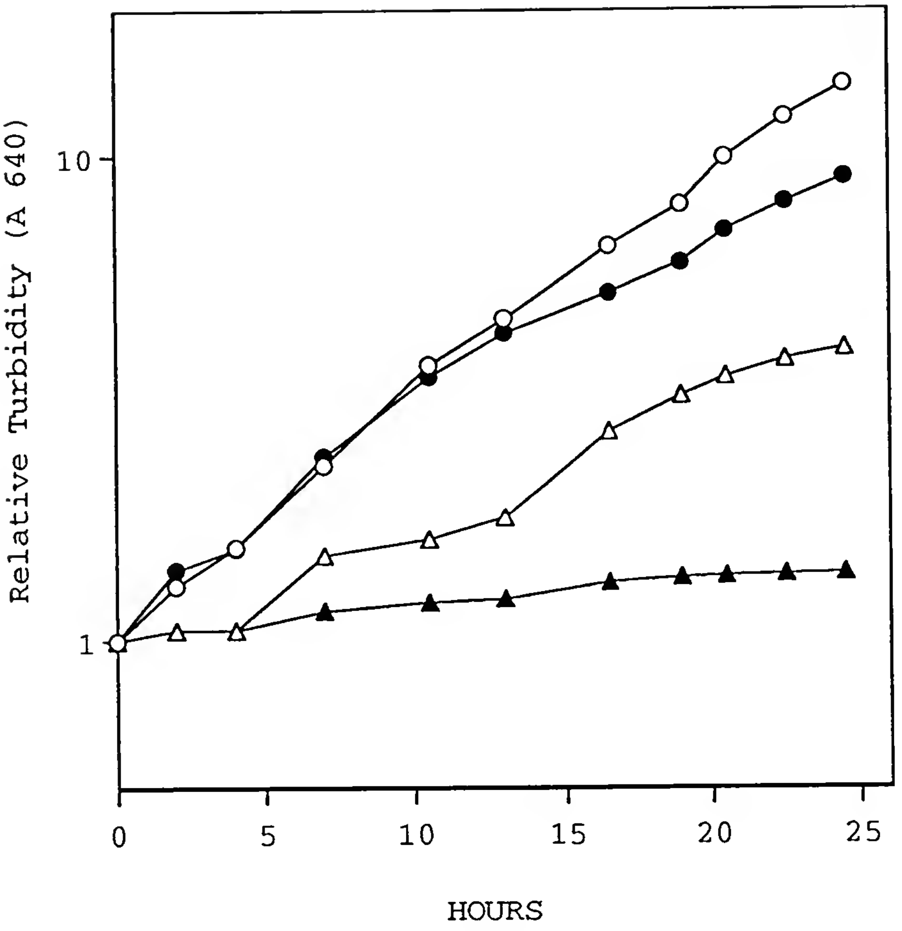

Relative Turbidity (A 640)
10
1
0
5
10
15
20
25
HOURS

Table 7. Effect of Norflurazon on growth rate, total carotenoid content, and percentage of acetolysis-resistant wall fraction of *C. sorokiniana*

Conditions	Doubling Time (Hours)	Total Carotenoids (µg/g dry weight of cells)	Acetolysis-resistant cell wall fraction (% cell wall dry weight)
Without Norflurazon	6.3	592 ± 95.0	23.6 ± 3.10
1 µM Norflurazon	7.1	171 ± 12.0	25.7 ± 0.400

neoxanthin, and ß-carotene (Kessler, 1961) (Table 8). Treatment of cells with norflurazon produced a uniform decrease in all carotenoid pigments. Decrease in carotenoid production was not reflected by decrease in amount of acetolysis resistant cell wall fraction.

Effect of MON-20763 on Cell Wall Synthesis

MON-20763 inhibits pollen exine formation presumably by inhibition of sporopollenin synthesis. To determine effects, if any, MON-20763 has on *C. sorokiniana* and *C. fusca* cell wall biosynthesis, these algae were cultured in medium containing various concentrations of MON-20763. Both *C. sorokiniana* and *C. fusca* cultures exhibited decreased growth in cultures containing MON-20763. *C. fusca* cultures continued to increase in turbidity at much higher concentrations of MON-20763 than did *C. sorokiniana* cultures (Figure 14). From TEM photographs, it appeared that the increase in culture turbidity (shown in Figure 14) resulted from cell enlargement instead of an increase in cell number (Figure 15). Multiple nuclei and chloroplasts were present in many MON-20763-treated cells. New daughter walls did not form between the nuclei and chloroplasts. The original wall (mother wall) did not break open and release partially-formed walless cells. Some breakage or fraying of outer walls was visible. Incubation of MON-20763-treated *Chlorella* cells in CHP did not enhance wall degradation (Figure 16). Since even without prior enzyme treatment, enlarged cells

Table 8. Identification of carotenoids from C. *sorokiniana* using thin layer chromatography and absorption spectrum analysis

	Published absorption maxima[a]		Experimental absorption maxima	
Carotenoid	in ethanol	in chloroform	in ethanol	in chloroform
Lutein	422, 445, 474	435, 458, 485	424, 445, 474	432, 455, 485
Violaxanthin	419, 440, 470	426, 449, 478	419, 442, 470	428, 452, 479
Neoxanthin	415, 439, 467	423, 448, 476	414, 437, 466	420, 445, 475
ß-Carotene (in acetone)	452, 478		451, 477	

[a] Britton (1991)

Figure 14. Growth of *C. sorokiniana* and *C. fusca* cells in cultures containing MON-20763. Solid symbols represent *C. sorokiniana* cultures and open symbols represent *C. fusca* cultures. The concentrations of MON-20763 in the cultures was; 0% (-Δ-), 1.2% (-□-), 2.4% (-O-), 0% (-▲-), 0.05% (-●-).

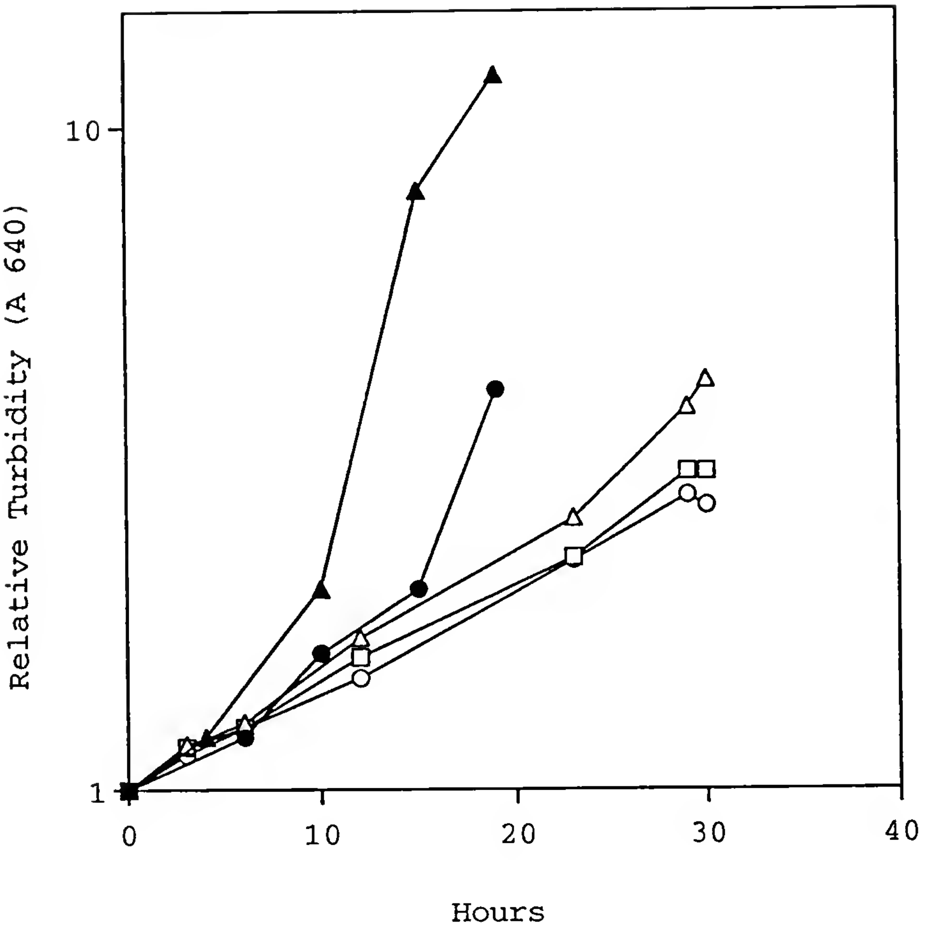

Relative Turbidity (A 640)
10
1
0
10
20
30
40
Hours

Figure 15. Morphology of *C. sorokiniana* and *C. fusca* cells grown in cultures containing MON-20763. Figures are as follows; *C. sorokiniana* grown without MON-20763 (a), *C. sorokiniana* grown with 0.05% MON-20763 (b), *C. fusca* grown without MON 20763 (c), *C. fusca* grown with 2.4% MON-20763 (d). Enlargement X12,500.

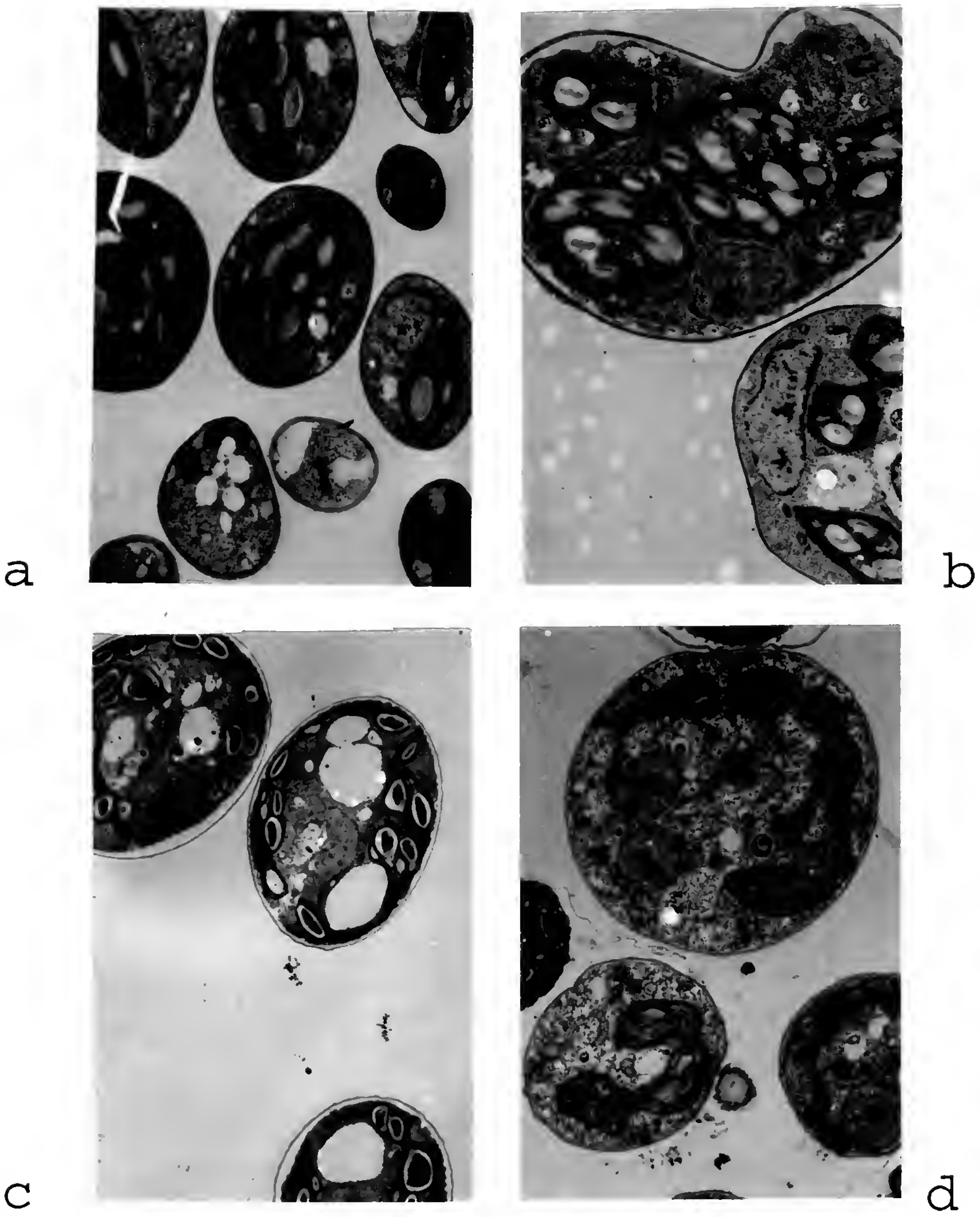
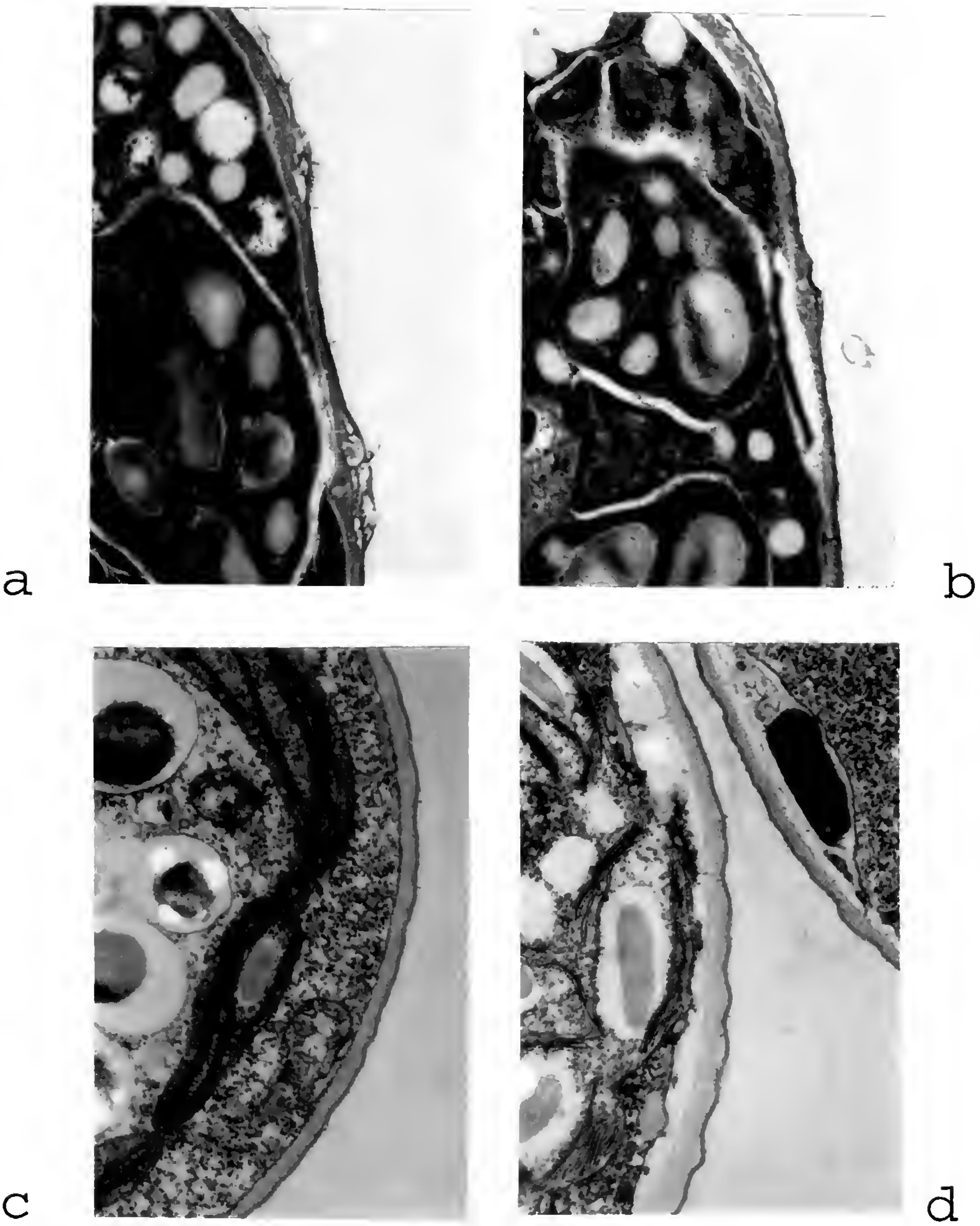

a
b
c
d

Figure 16. Wall morphology of *C. sorokiniana* and *C. fusca* cells grown in MON-20763 with and without subsequent CHP treatment. *C. sorokiniana* grown in 0.05% MON-20763 without CHP treatment (a), *C. sorokiniana* grown in 0.05% MON-20763 with CHP treatment (b), and *C. fusca* grown in 2.4% MON-20763 without CHP treatment (c), and *C. fusca* grown in 2.4% MON-20763 with CHP treatment (d). Enlargement X50,000.


a
b
c
d

were fragile and often broke under a coverslip, osmotic lability of these cells was difficult to assess.

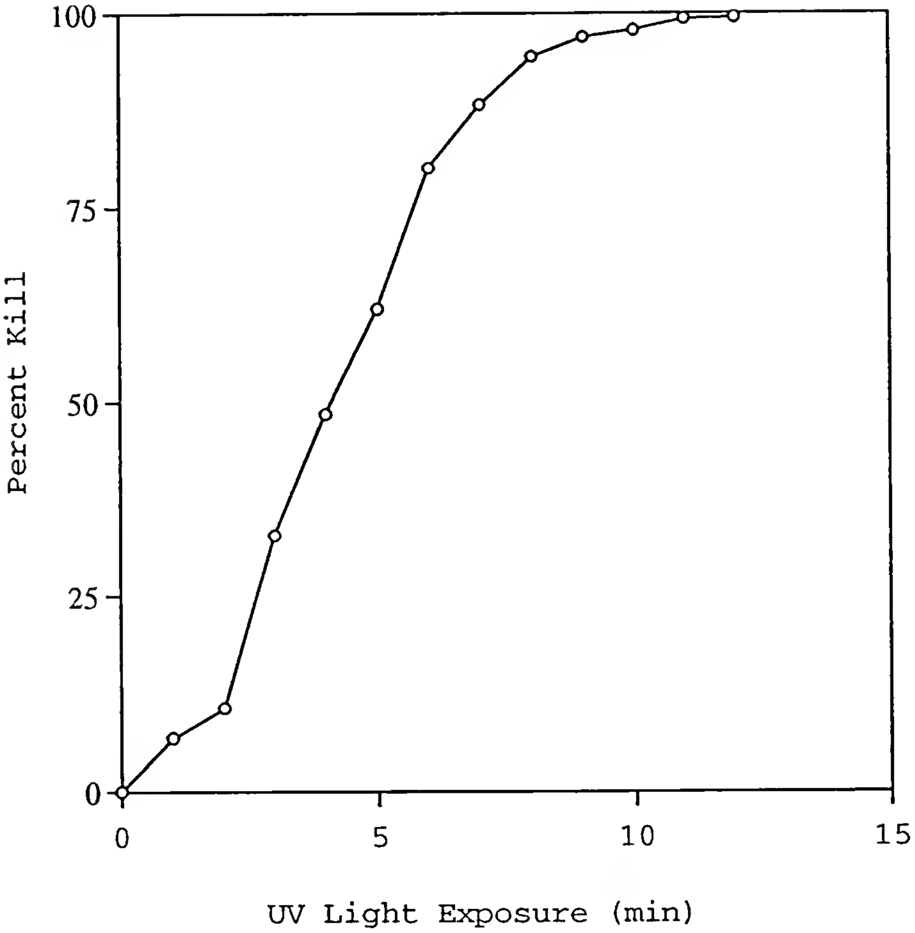
Creation, Selection, and Characterization of Cell Wall Defective Mutants

Rationale for the procedure to select cell wall defective mutants of *C. sorokiniana* cells was based upon an observation that intact wild-type cells of *C. ellipsoidea* and *C. saccharophila* were resistant to Nonidet P-40 (NP-40), whereas protoplasts of these cells were readily lysed by this detergent. Upon cell lysis of the protoplasts, chlorophyll was released into surrounding medium. Centrifugation of lysates revealed a green supernatant. *C. sorokiniana* cell wall defective mutants formed using UV mutagenesis could be screened by treatment with hydrolytic enzymes, exposure to NP-40, and examination for green supernatants. Walls with sensitivity to hydrolytic enzymes should produce green supernatants. Mutant walls that are altered in structure to allow detergent to come in contact with and disrupt cell membranes would also produce green supernatants.

Wild-type *C. sorokiniana* cells were insensitive to detergent treatment. UV irradiation and enzyme/detergent treatment did result in formation and identification of *C. sorokiniana* cell wall defective mutants.

Exposure of cell suspensions to UV light for 7 min and 45 s resulted in 97-98% kill (Figure 17). A total of 10,262

Figure 17. Kill curve of *C. sorokiniana* cells exposed to UV light. Twenty ml of 2 x 10^9 cells/ml were exposed to UV light for a total of 12 min. Aliquots of cells were harvested at 1 min intervals and spread on plates of SUN medium plus 50 mM glucose. Plates were incubated for 7 d in darkness prior to counting surviving colonies.

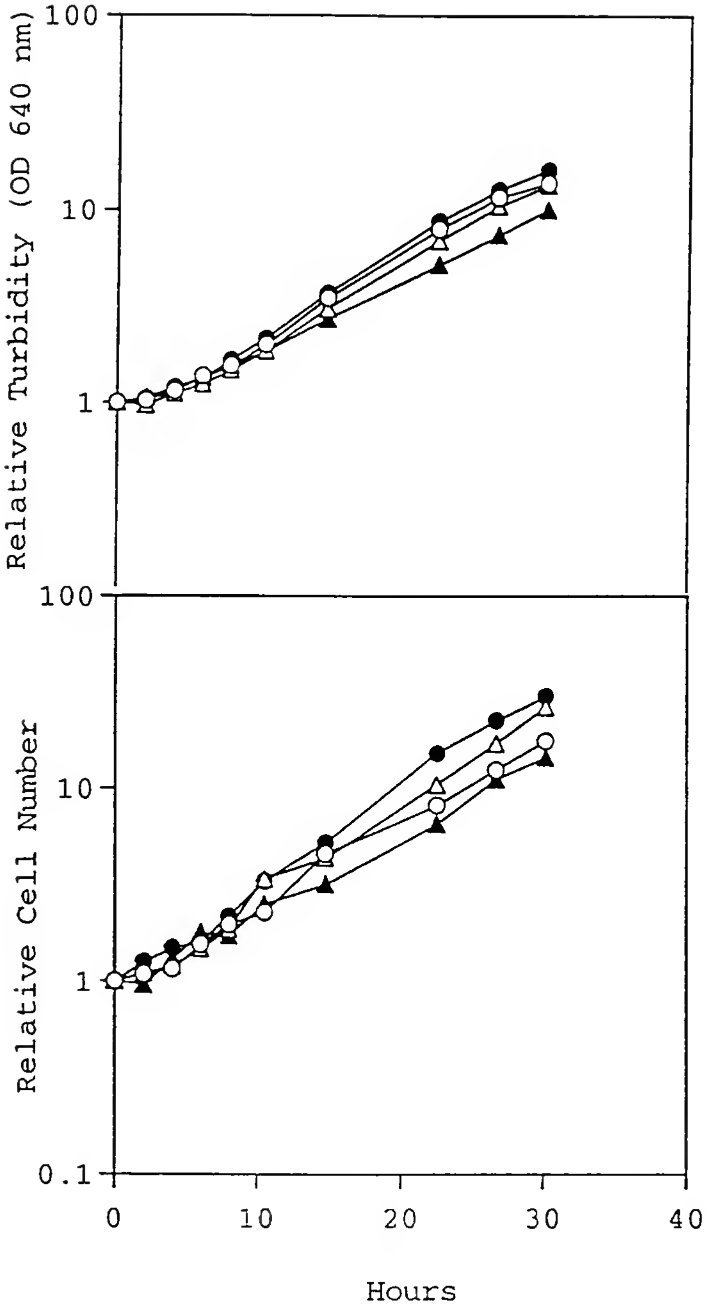

100
75
50
25
0
Percent Kill
0
5
10
15
UV Light Exposure (min)

colonies were subjected to the screening procedure for selection of cell wall defective mutants. From the 10,262 colonies, five colonies with a mutant phenotype were identified from which three stable mutants were recovered. The three mutants were designated as #2, #3, and #5. The phenotype of each mutant was stable for at least 8 months. When grown heterotrophically in the dark, mutants #2 and #3 increased in turbidity and cell number at a rate similar to the wild type. Mutant #5 cultures increased in turbidity and cell number at a rate slightly slower than the wild type (Figure 18).

In one experiment in which a *C. ellipsoidea* positive control was included, the mutants were grown autotrophically and did not produce a mutant phenotype; for this reason the remainder of mutant experiments were conducted with cells grown heterotrophically in darkness. CHP treatment and suspension of treated cells in NP-40 or Triton were both essential for the production of the mutant phenotype. The mutant phenotype was observed within 2.5 h after the start of enzyme treatment. In TEMs of the cells after 2.25 h of enzyme treatment (prior to exposure to a detergent), the cell membranes were poorly defined indicating signs of cell death or enzyme-induced damage.

A study was undertaken to determine if the mutant phenotype was caused by an enzymatic effect on cell walls or if a component, such as a filler or stabilizer added to the commercially prepared enzymes, was responsible for a decrease

Figure 18. Growth of wild type and mutant *C. sorokiniana* cells. Cells were grown in SUN medium supplemented with 50 mM glucose in the dark. Growth is presented as an increase in turbidity and an increase in cell number. The cultures are as follows; wild type (-O-), mutant #2 (-Δ-), mutant #3 (-●-), and mutant #5 (-▲-).


Relative Turbidity (OD 640 nm)
100
10
1
Relative Cell Number
100
10
1
0.1
0
10
20
30
40
Hours

in cell integrity. The CHP enzyme solution was separated by ultrafiltration into a fraction containing fillers and a fraction primarily containing enzymes. When treated with CHP, or CHP from which fillers had been removed, mutants, but not the wild type cells, had a mutant phenotype. When treated with solution containing fillers but no enzyme, only mutant #5 faintly exhibited a mutant phenotype.

Compositional analysis was performed on purified mutant cell walls. Monosaccharides identified in TFA and HCl extracts of wild type and mutant walls were identical. Rhamnose, galactose, and glucuronic acid composed over 50% of the dry weight of each cell wall type.

TFA was used to hydrolyze cell wall matrix polysaccharides. Approximately 50% of cell wall dry weight was extracted with TFA from both wild type and mutant walls (Table 9). Like the wild type, mutant TFA extracts consisted primarily of rhamnose and galactose. Cell walls of mutants #2 and #3 had slightly lower rhamnose and slightly higher galactose than those of the wild type; whereas, mutant #5 had less rhamnose and about twice the amount of galactose as the other strains (Figure 19). Remaining monosaccharides in mutant cell walls were present in concentrations similar to the wild type.

The same monosaccharides were identified in HCl hydrolysates for each of the wild type and mutant strains (Figure 20). Greater variation in quantitative analysis of cell wall monosaccharides was present in HCl hydrolysates

Table 9. Comparison of *C. sorokiniana* wild type and mutant TFA-extractable cell wall monosaccharides

	Percentage of Cell Wall Dry Weight			
		Mutant Number		
Monosaccharide	Wild Type	#2	#3	#5
Rhamnose	38	33	30	24
Xylose	0.75	0.80	0.80	0.70
Xylitol	1.8	1.6	2.0	1.5
Mannose	2.1	2.4	2.3	1.8
Galactose	6.7	8.2	9.5	18
Glucose	0.45	0.22	0.31	0.44
Glucuronic Acid	2.6	2.7	3.2	2.6
Glucosamine	0.10	0.2	0	0
Galactosamine	0	0	0	0
Total Polysaccharide[a]	50	47	46	47

[a] Total polysaccharide was estimated by calculating mol of monosaccharide and subtracting the weight of one mol of water lost upon formation of a glycosidic bond for every two mol of monosaccharide.

Figure 19. Comparison of monosaccharide composition of TFA hydrolysates of wild type and mutant *C. sorokiniana* cell walls. Walls were hydrolyzed in 2 M TFA for 6 h at 100°C. After evaporation of TFA, residues were analyzed using GC-MS.

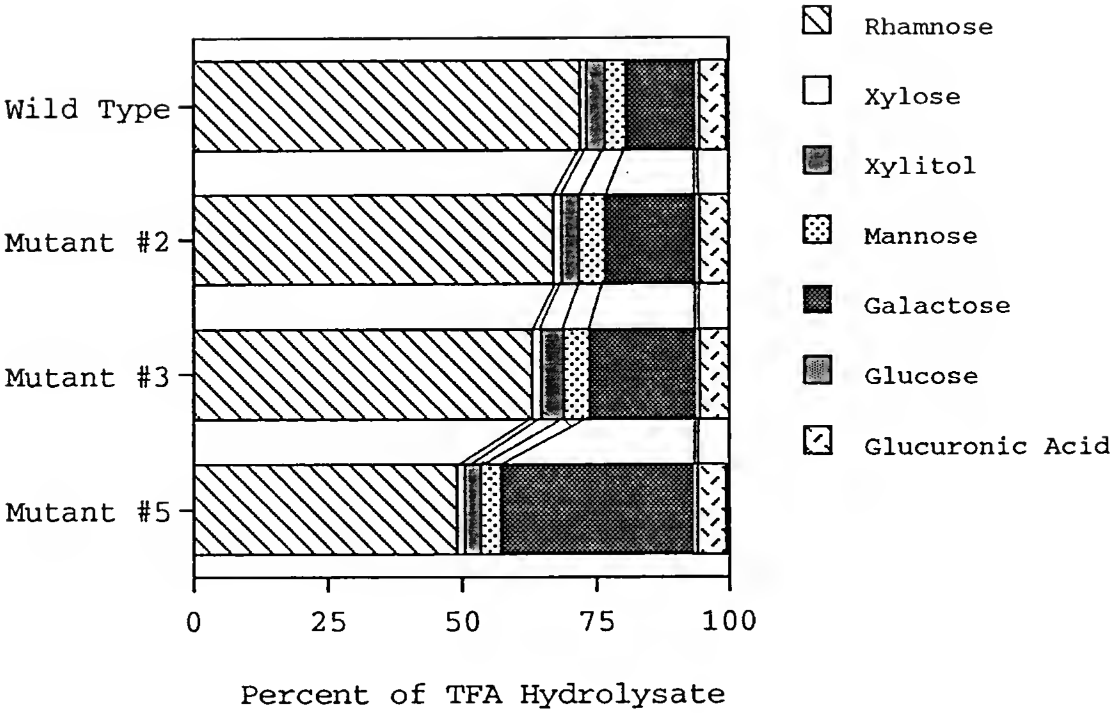
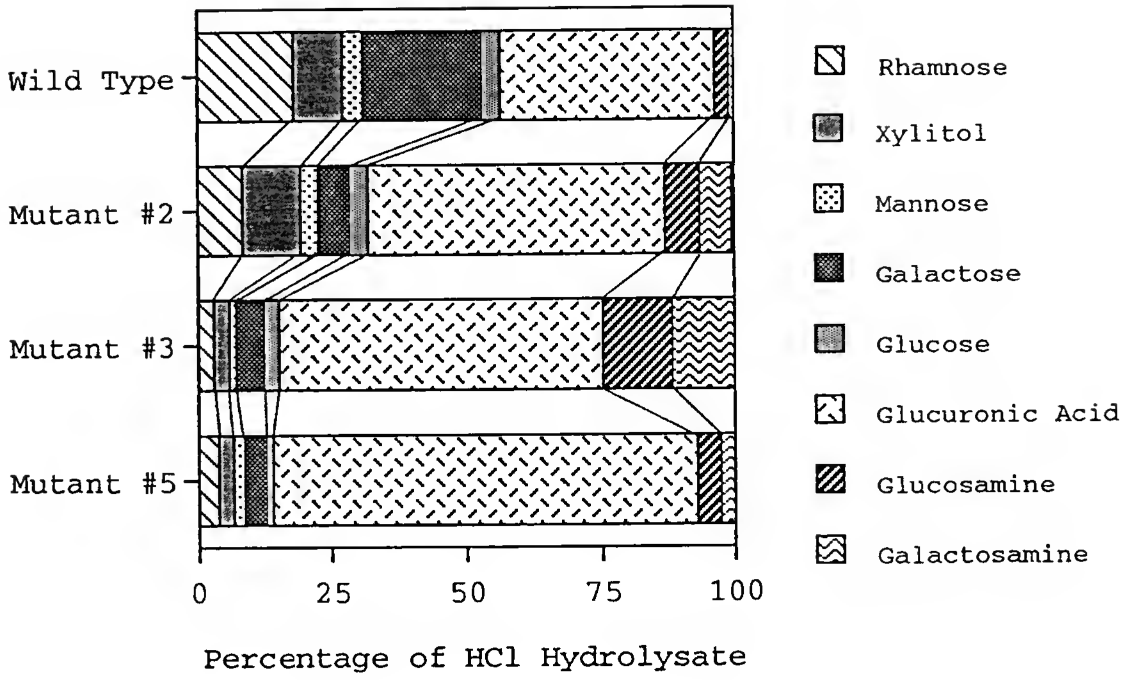

Wild Type
Mutant #2
Mutant #3
Mutant #5
0
25
50
75
100
Percent of TFA Hydrolysate
Rhamnose
Xylose
Xylitol
Mannose
Galactose
Glucose
Glucuronic Acid

Figure 20. Comparison of monosaccharide composition of HCl hydrolysates of wild type and mutant *C. sorokiniana* cell walls. Purified cell walls were treated with 6 N HCl for 18 h at 110°C. After evaporation of the HCl, residues were analyzed using GC-MS.


Wild Type
Mutant #2
Mutant #3
Mutant #5
0
25
50
75
100
Percentage of HCl Hydrolysate
Rhamnose
Xylitol
Mannose
Galactose
Glucose
Glucuronic Acid
Glucosamine
Galactosamine

than in TFA hydrolysates. A total of 13-35% of wall dry weight of each mutant or wild type strain was hydrolyzed with HCl (Table 10). Glucuronic acid was the primary monosaccharide extracted with HCl from wild type and mutant walls. Percentage of cell wall dry weight of glucuronic acid released varied from 8.3% for mutant #3 to 29% for mutant #5 as compared to 14% for the wild type. Amount of rhamnose and galactose extracted from wild type walls was slightly higher than it was for mutant walls.

Amino sugars in HCl hydrolysates accounted for 1.7%-3.6% of cell wall dry weight of each strain. Glucosamine accounted for less than 2% of any wall tested. The gas chromatogram for each cell strain had a peak representing an unidentified amino sugar. Since this monosaccharide was not identified, its concentration could not be determined. Amount of this unidentified amino sugar is not calculated into the reported total for amino sugars.

Combined data for both TFA and HCl hydrolysates were qualitatively identical for the wild type and mutant strains (Figure 21). About 83% of the wild type wall but only 59% of mutant #3 wall was recovered in acid hydrolysates (Table 11). Low monosaccharide yield for #3 resulted mostly from low amounts of galactose, glucuronic acid, and xylose extracted by HCl hydrolysis. Greater than 50% of the wall dry weight of each cell type consisted of rhamnose, glucuronic acid, and galactose. However, the wall of each mutant strain had less rhamnose and galactose than did the wild type. Mutant cell

Table 10. Comparison of *C. sorokiniana* wild type and mutant HCl-extractable cell wall monosaccharides

	Percentage of Cell Wall Dry Weight			
		Mutant Number		
Monosaccharide	Wild Type	#2	#3	#5
Rhamnose	6.2	2.4	0.40	1.4
Xylose	0	0	0	0
Xylitol	3.3	3.3	0.40	0.99
Mannose	1.3	0.96	0.17	0.72
Galactose	7.6	1.7	0.74	1.5
Glucose	1.2	0.99	0.41	0.47
Glucuronic Acid	14	16	8.3	29
Glucosamine	0.92	1.9	1.8	1.7
Galactosamine	0.68	1.7	1.6	0.98
Total Polysaccharide[a]	33	28	13	35

[a] Total polysaccharide is estimated by calculating mol of monosaccharide and subtracting weight of one mol of water lost upon formation of a glycosidic bond for every two mol of monosaccharide.

Figure 21. Comparison of monosaccharides in total acid hydrolysates of wild type and mutant *C. sorokiniana* cell walls. The quantity of each monosaccharide presented represents total monosaccharide released from both TFA and HCl hydrolysis.

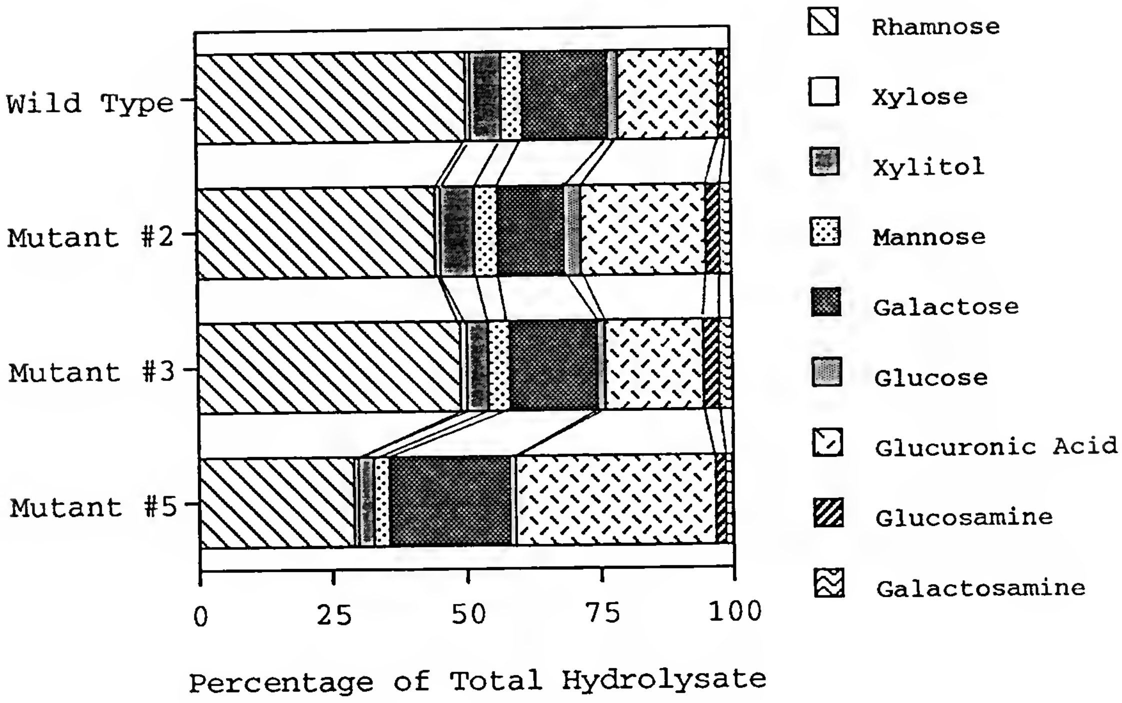

Wild Type
Mutant #2
Mutant #3
Mutant #5
0
25
50
75
100
Percentage of Total Hydrolysate
Rhamnose
Xylose
Xylitol
Mannose
Galactose
Glucose
Glucuronic Acid
Glucosamine
Galactosamine

Table 11. Comparison of *C. sorokiniana* wild type and mutant total extractable cell wall monosaccharides

	Percentage of Cell Wall Dry Weight			
		Mutant Number		
Monosaccharide	Wild Type	#2	#3	#5
Rhamnose	44	35	30	25
Xylose	0.75	0.80	0.80	0.70
Xylitol	5.1	4.9	2.4	2.5
Mannose	3.4	3.4	2.5	2.5
Galactose	14	9.9	10	19
Glucose	1.7	2.4	0.85	0.92
Glucuronic Acid	17	19	12	32
Glucosamine	1.0	2.1	1.8	1.7
Galactosamine	0.68	1.7	1.6	0.98
Total Polysaccharide[a]	83	75	59	81

[a] Total polysaccharide was estimated by calculating mol of monosaccharide, and subtracting the weight of one mol of water lost upon formation of a glycosidic bond for every two mol of monosaccharide.

walls had slightly higher cell wall protein concentrations than did wild type walls (Table 12). As reported for wild type analysis, the Bradford assay consistently estimated cell wall protein concentration to be about 53% less than did the Lowry assay or the amino acid analyzer. Amino acid analysis of cell wall proteins of each mutant was similar to that of the wild type (Table 13). Hydroxyproline was present in very low quantities. Glycine and alanine were present in concentrations slightly higher than other amino acids. Percentage of cell wall dry weight estimated as polysaccharide and protein combined in cell walls of each mutant and wild type strain is 100%, 97%, 81%, and 100% for the wild type, #2, #3, and #5, respectively.

Table 12. Comparison of *C. sorokiniana* wild type and mutant cell wall protein concentrations

Protein Determination Method	Percentage Cell Wall Dry Weight			
	Wild Type	#2	#3	#5
Bradford[a]	9.0 ± 0.72	13 ± 1.1	13 ± 1.0	11 ± 0.46
Lowry[a]	17 ± 1.4	24 ± 1.9	24 ± 1.4	20 ± 0.64
Amino acid analyzer[b]	17	22	22	19

[a] Total protein was extracted from purified cell walls with 1 N NaOH for 20 h at 37°C.

[b] Amino acids were extracted from purified cell walls with 6 N HCl, 1% phenol, and 0.02% mercaptoethanol for 20 h at 110°C.

Table 13. Comparison of *C. sorokiniana* wild type and mutant cell wall protein amino acid composition

	mol %			
		Mutant Number		
Amino Acid	Wild Type	#2	#3	#5
Hyp	0.020	0.020	0.030	0.040
Asx	8.33	8.51	8.59	8.68
Thr	6.59	6.07	6.18	6.33
Ser	3.74	4.20	4.12	3.94
Glx	5.63	6.65	6.54	6.21
Pro	6.60	6.35	6.47	6.94
Gly	7.90	9.42	9.26	8.86
Ala	8.18	10.5	10.4	9.50
Val	6.99	7.56	7.43	7.01
Met	0.69	1.22	1.10	0.78
Ile	3.60	4.01	3.87	3.61
Leu	5.26	7.26	6.80	5.91
Tyr	6.45	4.79	4.90	5.43
Phe	6.30	5.53	5.65	6.18
His	3.67	2.96	3.28	3.50
Arg	5.93	5.25	5.28	5.08

DISCUSSION

The *C. sorokiniana* cell wall is composed of polysaccharides and proteins. Combined amounts of polysaccharide and protein account for approximately 100% of cell wall dry weight. It is unlikely that significant quantities of other compounds are present in this wall. Therefore, polysaccharide and/or protein components of the wall are likely to be responsible for observed cell wall autofluorescence, resistance to acetolysis, and resistance to degradation by enzymes.

With one exception, individual monosaccharides identified by Takeda (1991), in 2 M TFA hydrolysates for *C. sorokiniana*, are consistent with those presented here. The major matrix monosaccharides are (from highest to lowest concentration) rhamnose, galactose, mannose, xylitol, and xylose. Presence of xylitol was not reported by Takeda whose GC analysis was performed on alditol acetate derivatives of each monosaccharide. Formation of alditol acetate derivatives prevents distinction between xylitol and xylose by gas chromatography. In the data shown here, GC analysis was performed on TMS derivatives which allows for distinction between monosaccharides and their corresponding alditols. Occurrence of xylitol as a metabolic intermediate is well documented. Although, previous reports of xylitol as a constituent of cell wall polysaccharides have not been found.

Glucosamine was the only monosaccharide previously reported to be in 6 N HCl hydrolysates (the rigid portion of the wall) of walls of three strains of *C. sorokiniana* (Takeda, 1991). In GC-MS data presented here, glucosamine accounted for only about 2% of the HCl hydrolysate. Other monosaccharides in the HCl hydrolysate were (from highest to lowest concentration) galactose, rhamnose, xylitol, mannose, glucose, and galactosamine. The only rigid wall polysaccharides reported in the literature for any *Chlorella* species contain glucose and mannose or glucosamine.

In the present study, an unknown amino sugar was found by GC-MS. It was observed to have a peak area larger than any other monosaccharides identified in the HCl hydrolysate. However, until a known standard can be identified that has the same retention time and mass to charge ratio as the unknown compound, identity and concentration of this compound can not be determined. If the response factor of the unknown compound is relatively high, which is the case for glucosamine, the unknown compound may represent only a minor part of the HCl hydrolysate. The possibility that the peak is actually an artifact has not been ruled out (Chris West, personal communication).

Differences in methods used for monosaccharide identification may account for qualitative differences between data reported here and by Takeda (1991) for the *C.*

sorokiniana wall composition. Takeda (1991) used a combination of gas chromatography and thin layer electrophoresis to identify neutral sugars and amino sugars. In the experiments reported here, monosaccharides were identified using GC-MS.

In addition to GC-MS analysis, *C. sorokiniana* cell wall HCl hydrolysates were also analyzed using spectrophotometric assays for total carbohydrate, glucose, and amino sugars. Data from spectrophotometric assays were not consistent with those from GC-MS analysis. Approximately 31% of cell wall dry weight, measured as monosaccharides by GC-MS, should have also been detected by the phenol sulfuric acid assay for total carbohydrate (Table 1) (amino sugars are not quantified by the phenol sulfuric acid assay). Less than 1% of the cell wall dry weight was detected as carbohydrate using the spectrophotometric assay (Table 2). These data are consistent with those previously reported for *Chlorella* in that only amino sugar(s) were detected in HCl hydrolysates. Also, amount of amino sugar measured using the Elson-Morgan assay is consistent with amount of glucosamine quantified using GC-MS. This observation suggests that the unknown amino sugar detected using GC-MS does not contribute substantially to cell wall dry weight. Also, if the unknown sugar was present at a high concentration, a higher amount of amino sugar would have been measured using the Elson-Morgan assay for amino sugars than was measured using GC-MS.

Uronic acid concentrations are the only data on *C. sorokiniana* cell wall monosaccharide composition that has been previously reported quantitatively. Takeda (1991) reported that uronic acid, quantified by the carbazole assay, in 2 M TFA hydrolysates of two strains of *C. sorokiniana* comprised 11.9% (strain 211-8k) and 8.9% (strain 211-32) of cell wall dry weight. In data presented herein, 2 M TFA extracted 1.9% of the cell wall dry weight as uronic acid as measured by the metahydroxy diphenyl assay (this assay has less interference from hexoses than does the carbazole assay) and 2.6% glucuronic acid by GC-MS. An additional 14% glucuronic acid in the 6 N HCl hydrolysate was quantified by GC-MS, for a total of approximately 17% of cell wall dry weight composed of uronic acid. Glycosidic linkages of uronic acids are fairly acid resistant; therefore, a quantitative yield of monosaccharides is not typically obtained using TFA. More severe conditions for hydrolysis of polysaccharides containing uronic acids are not used because uronic acid monosaccharides are labile in hot acid (Fry, 1988). Since uronic acids should not survive 6 N HCl treatment, it is unclear why such a high recovery of uronic acid was obtained. Although most species of *Chlorella* have lower concentrations of uronic acid in 2 M TFA hydrolysates than that reported here for *C. sorokiniana*, two strains of *C. saccharophila* var. *ellipsoidea* are reported to have up to 23.6% uronic acid in the wall (Takeda, 1991). If HCl hydrolysates also would have been analyzed by Takeda (1991),it is possible that he would have observed a higher uronic acid concentration than reported.

Approximately 17% of the *C. sorokiniana* cell wall dry weight is protein. This amount of protein is relatively high, but within the range of what has been reported for other species of *Chlorella*. Calculation of total protein from cell wall alkaline and acid extracts resulted in similar protein concentrations (Table 12). Three symbiotic strains of *Chlorella*, with walls similar to *C. sorokiniana*, contain 6.4-10% cell-wall protein (Kapaun et al., 1992). As shown in the data presented (Table 13), hydroxyproline makes up only about 0.02 mol% of the amino acids extracted from the *C. sorokiniana* wall. Because many cell wall proteins that display elicitor-induced antimicrobial (enzyme-inhibitory) activities are hydroxyproline-rich glycoproteins, an insignificant amount of hydroxyproline is notable (Bradley et al., 1992).

Glucose in the H_2SO_4 hydrolysate accounted for less than 1% of the cell wall dry weight. Therefore, cellulose is not a major cell wall polysaccharide. The *C. sorokiniana* wall is reported to have positive anisotropy (Takeda, 1991), which predicts a high degree of crystallinity. However, at present, it is not known what wall-component contributes to its crystalline structure. Cellulose and chitin are present in cell walls in the crystalline form. However, chitosan found in some fungal walls is not believed to be crystalline (Ruiz-Herrera, 1992).

There is evidence that glucosamine in the *C. sorokiniana* cell wall is present as chitosan and is a structural polysaccharide. Other possibilities are that glucosamine is a component of chitin or glycoprotein. The *C. ellipsoidea* C-27 cell wall is partially solubilized by chitosanase but not by chitinase (Satoh and Takeda, 1989). Using an enzyme mixture in which chitosanase was an essential component, osmotically labile *C. sorokiniana* cells were inconsistently prepared. The alkaline-insoluble cell wall residue had approximately a 7:1 carbon:nitrogen ratio. Since protein and glucosamine are the only nitrogen-containing compounds identified in the wall, and protein is extracted with alkali, it is likely the alkaline-insoluble wall residue consists largely of glucosamine. Also, an IR spectrum of alkaline-insoluble cell wall residue closely resembles that of authentic chitosan indicating that the *C. sorokiniana* alkaline-insoluble wall fraction is composed of a compound very similar to chitosan.

There is no evidence that compounds other than polysaccharide and protein are present in the *C. sorokiniana* wall. Wall morphology is inconsistent with trilaminar morphology of algal walls containing sporopollenin. In transmission electron micrographs, the *C. sorokiniana* cell wall consists of either a single homogeneous layer or a two-layered wall with single thin outer electron-dense layer. Two cell-wall layers exist and become more apparent when the cell walls are loosened. Wall loosening is apparent in TEM photos of mother walls free in the medium and MON-20763-treated cells. A thin outer layer is also more apparent in the MON-20763-treated walls where cell wall structure is severely disrupted. Inconsistency in ability to distinguish distinct wall layers on TEM photographs was also reported for the four-layered *Saccharomyces cervisiae* ascospore wall (Briza et al., 1988).

It is unlikely that the acetolysis-resistant portion of the *C. sorokiniana* cell wall is derived from carotenoids. Inhibition of total carotenoid biosynthesis in *C. sorokiniana* by 71% had no effect on amount of acetolysis-resistant residue in whole cells.

Chemical tests and spectroscopic analyses were used to confirm absence of both sporopollenin and lignin in the *C. sorokiniana* wall. Sporopollenin is characterized based upon its solubility. Solubility of the *C. sorokiniana* wall in phosphoric acid indicates sporopollenin is not a component of this algal wall. A negative qualitative phloroglucinol test indicated that lignin was not a cell wall component. Phenolic compounds were detected when alkaline extracts of intact walls were analyzed by NMR and UV absorption spectroscopy. Moreover, an IR spectrum of whole walls did not reveal presence of phenolic compounds.

C. sorokiniana cell wall synthesis was inhibited by a pollen biosynthesis inhibitor, MON-20763. In most cases, when cells were grown in MON-20763, *C. sorokiniana* and *C. fusca* cell diameters more than doubled (Figures 14 and 15).

Enlarged cells exhibited chloroplastic and nuclear division, as if cells were preparing to divide. MON-20763 arrested cell development prior to formation of cell membranes and walls around each daughter cell. Cell division did not occur. Two hypotheses as to a mechanism of MON-20763 inhibition of pollen synthesis are that this inhibitor (i) prevents polymerization of sporopollenin monomers (El-Ghazaly and Jensen, 1990) or (ii) prevents transport of sporopollenin monomers from cytoplasm to cell wall (Schulz et al., 1993). In higher plants, cell membrane lipids and proteins, and cell wall matrix polysaccharides and proteins are synthesized cytoplasmically and transported to the cell wall in cytoplasmically-derived vesicles. A mechanism common to transport of pollen sporopollenin and *Chlorella* cell membrane and cell wall components from cytoplasm to pollen or algal wall is most likely inhibited by MON-20763.

Despite the inhibition of daughter cell-wall synthesis, protoplasts could not be made from the MON-20763-treated *C. sorokiniana* cells. The mother wall was still intact, and was not affected by enzymatic or acetolysis treatments. In electron micrographs, damage to the wall is evident by broken and frayed areas of the cell wall. Since loosening of a wall would allow enzymes greater accessibility to the substrate, one would expect the mother wall to be more sensitive to enzymatic digestion. Increased digestion of the damaged wall by polysaccharide-degrading enzymes did not occur. Lack of complete digestion of the cell wall by polysaccharide-degrading enzymes (cellulase, hemicellulase, and pectinase) indicates that it is a lack of a specific degradative enzyme(s), and not a compact wall structure that prevents efficient degradation of the *C. sorokiniana* wall.

A purified cell wall with an enzyme-resistant outer layer and a digestible inner layer, would be expected to be partially degraded when exposed to enzymes. It is unlikely that digestion of the *C. sorokiniana* wall is being prevented solely by a resistant outer layer. Only 6% of purified cell wall dry weight was released as carbohydrate. Also, when thin sections of cells were treated with CHP, the inner cellulosic layer of the *C. fusca* cell wall was apparently degraded as indicated by a decrease in electron density. A similar change in the *C. sorokiniana* wall was not observed. In an attempt to document the effect(s) of cell homogenate, protease, CHP, and chitosanase on the *C. sorokiniana* cell wall, a similar experiment was conducted. A decrease in electron density was not observed for the *C. sorokiniana* cell wall. Since the experiment was performed with an enzyme mixture that did not produce osmotically labile cells, results of treatment of *C. sorokiniana* thin sections with the second enzyme solution should be considered preliminary. For both enzyme treatments, negative results may not be definitive. Biological molecules have different affinities for stains that are used for post-staining in electron microscopy. If one cell wall compound is degraded, it may not be apparent in electron micrographs if it is associated with compounds that are more electron dense.

Imines (Schiff bases), which are highly fluorescent, are formed upon reaction of a primary amine with an aldehyde. Volatile aldehydes produced by lipid oxidation have been shown to react with chitosan resulting in chitosan fluorescence (Weist and Karel, 1992). *C. sorokiniana* cell wall autofluorescence is most likely due to formation of imines at primary amino groups of chitosan. Imines may also form at primary amines of N-terminal amino acids, lysine, and arginine. Aromatic groups of phenylalanine and tyrosine have intrinsic autofluorescence and may also contribute to cell wall autofluorescence. Chitosan, sold as "purified" chitosan, has autofluoresce. A considerable amount of autofluorescent compound(s) was extracted from the *C. sorokiniana* cell wall with 1 N NaOH. This extraction also removes cell wall protein. The acetolysis resistant portion of the wall was also autofluorescent and had a C:N ratio of 5:1.

Much of the resistance of the *C. sorokiniana* wall to acetolysis is likely to result from presence of a large amount of rhamnose in the wall. Polysaccharides containing rhamnose and other 6-deoxy sugars such as fucose, are fairly resistant to acetolysis. About 22% of the cell wall dry weight is resistant to acetolysis; 38% of the wall dry weight is rhamnose. After TFA hydrolysis, the cell wall dry weight has about 8.4% acetolysis resistant residue and 6.2%

rhamnose. Alkaline extraction of the wall, which also removes rhamnose, decreased the acetolysis resistant residue to less than 1% of cell wall dry weight.

Cell wall residue that remains following alkaline extraction and acetolysis has an IR spectrum that resembles the whole cell wall. However, the residue does not have a distinct spectrum that allows for identification of a single compound. Because the resistant residue has a C:H:O:N ratio of 5:8:2:1 (oxygen is calculated to bring the total wall mass to 98% C, H, O, and N) and rhamnose does not contain nitrogen, it is unlikely that the resistant residue consists of only rhamnose. A high C:N ratio and autofluorescence of the acetolysis-resistant residue suggests that the residue is derived from chitosan or protein.

It appears that protein contributes largely to resistance of the wall to degradation by polysaccharide-degrading enzymes. Although approximately 87% of the *C. sorokiniana wall* is composed of polysaccharide, only about 6% of the wall dry weight was released as carbohydrate after treatment of purified cell walls with cellulase, hemicellulase, and pectinase. Amount of carbohydrate released was not enhanced by treatment with chitosanase. The only *Chlorella* species from which protoplasts could be made and cell-wall carbohydrate and protein data were reported was *C. saccharophila* 211-1a. *C. saccharophila* has a wall with 14% uronic acid, and 1.7% protein, and a glucose/mannose rigid portion (Blumreisinger et al., 1983; Gobel and Aach, 1985).

In a previous study, three species of *Chlorella*, having glucosamine rigid walls, had cell wall autolytic activities described as proteolytic (Araki and Takeda, 1992). Protoplasts were produced from *C. ellipsoidea* cells using an enzyme mixture that contained, in addition to mixed glycosidases, a cell homogenate and a chitosanase (Hatano et al., 1992). Both homogenate and protease had proteolytic activities. In this dissertation research, treatments tested on *C. sorokiniana* cells yielded (in some experiments) osmotically labile cells within 4 h suggesting that some cell wall degradation had occurred. In experiments in which osmotically labile cells were obtained, either *C. sorokiniana* cell homogenate or a protease was essential for the degradation of the cell wall. From these results, it appears to be essential to degrade the cell wall protein to allow for the formation of osmotically labile cells.

Following treatment of *C. sorokiniana* cells with cellulase, hemicellulase, pectinase, chitosanase, and either cell homogenate, protease, or both, osmotically labile cells were inconsistently produced. The reason(s) for inconsistent formation of osmotically labile cells remains unclear. Using the same starting culture for three consecutive experiments, osmotically labile cells were produced. In a fourth experiment the same starting culture was used, but a new bottle of pectinase was used (same company and catalog number, different lot number). Osmotically labile cells were not produced. Osmotically labile cells were not produced in six subsequent experiments.

Using the same starting culture as was used in the preceding successful experiments, osmotically labile cells were not produced in a fourth experiment. Therefore, it is unlikely that there was a contaminating organism in the culture that produced an enzyme that contributed to cell wall degradation. It is possible that the specific lot of pectinase had an enzymatic contaminant necessary for cell wall degradation. However, treatment of cells with an enzyme mixture containing new pectinase from two sources did not result in production of osmotically labile cells. Pectin is often difficult to degrade, and activities of pectinases from different sources vary. It is unlikely that cell growth stage influenced the lack of wall degradation. It has been reported in various species of *Chlorella* that growth stage of cells has an effect on protoplast yield (Braun and Aach, 1975; Berliner, 1977). In experiments presented herein, in which osmotically labile cells were produced, cells were only partially synchronized. Logarithmically-growing asynchronous cultures were stored for 16 h to 3 d under light without shaking. This treatment allows for a majority of cells to divide once (prior to 16 h) then stop growing. A starting culture, which consisted mostly of daughter cells, was grown autotrophically for either 3 or 4 h. Partially synchronized cells were used in subsequent enzyme treatments. Optimal synchronization requires culturing daughter cells through a series of three light/dark periods. Despite attempts to duplicate conditions of the first three experiments, in subsequent experiments, osmotically labile cells were not produced.

Five *C. sorokiniana* mutants displaying cell wall-defective phenotypes were identified. Three mutants were recovered and labeled as mutants #2, #3, and #5. Acid hydrolysates of mutant cell walls were analyzed by GC-MS and shown to contain monosaccharides identical to those of wild type walls. Mutant #2 cell walls had slightly lower rhamnose and galactose than wild type walls. Mutant #3 cell walls had a lower percentage of total carbohydrate than wild type walls as reflected by lower levels of glucuronic acid, rhamnose, xylitol, and galactose concentrations in HCl hydrolysate. Mutant #5 cell walls had a higher percentage of glucuronic acid and galactose and lower rhamnose than wild type cell walls. Walls of each mutant strain had slightly higher protein percentage than those of the wild type. Amino acid profiles of total wall protein were very similar for all mutant and wild type strains. Protoplasts could not be prepared from any mutant cells and all had acetolysis-resistant walls.

Mutant #5 had more distinct differences from wild type cells than did cells of mutants #2 and #3. Mutant #5 cells not only had high galactose, low rhamnose, and high glucuronic acid, as compared to the wild type cells, it had a slightly slower growth rate. Even when exposed to the "filler" fraction (mostly carbohydrate) of commercially-prepared enzymes, mutant #5 weakly displayed the mutant phenotype. It is possible that the mutant phenotype may be a result of wall degradation and/or osmotic stress.

It is unclear how alterations in cell wall composition of mutant cells could cause a cell wall defective phenotype. The detergent-sensitive phenotype of these mutants occurred only after treatment of cells with cellulase, hemicellulase, and pectinase. A change in polysaccharide composition or structure (i.e., degree of branching, hydrogen bonding of wall constituents, etc.) may have occurred allowing greater accessibility of hydrolytic enzymes to cell wall substrates.

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BIOGRAPHICAL SKETCH

Brenda L. Russell was born in Bryn Mawr, PA, on February 26, 1961. She graduated from Downingtown High School in 1979 where, as a senior, she was selected Outstanding Biology Student. She entered Bloomsburg State College (now Bloomsburg Univeristy of Pennsylvania) in Bloomsburg, PA, in August 1979 and graduated with a Bachelor of Arts in Biology degree in August 1983. While attending Bloomsburg State College, she conducted research with the Pennsylvania Department of Agriculture. Ms. Russell worked for the Pennsylvania Department of Agriculture and subsequently the United States Department of Agriculture until summer 1985 when she aquired her first full time research position at Walter Reed Army Institute of Research, Washington, D.C. From August 1985 to August 1987 she attended Virginia Polytechinc Institute and State University where she aquired a Master of Science degree in Microbiology. She worked as a biological scientist in the Department of Entomology and Nematology at the University of Florida and for the United States Department of Agriculture both in Gainesville, FL. In May 1990, she began her studies towards the Doctor of Philosophy degree in the Department of Microbiology and Cell Science at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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